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(54) Title: NUCLEIC ACID DETECTION METHOD AND SYSTEM

(57) Abstract: The present invention provides novel nucleic acid detection methods based on nucleic acid template-directed primer extension assays. Also provided are kits and genetic analysis methods. Included are microsequencing methods comprising detecting an incorporated nucleotide by measuring direct fluorescence intensity, fluorescence-based nucleic acid detection methods using oligonucleotide primers hybridizing to both complementary strands of a nucleic acid sample, and methods for decreasing background signal in nucleic acid template-directed primer extension assays.

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NUCLEIC ACID DETECTION METHOD AND SYSTEM

FIELD OF THE INVENTION

The present invention relates to nucleic acid detection. The invention relates to methods and kits for detection nucleic acids, particularly useful for the detection of biallelic markers in the field of DNA genotyping.

BACKGROUND

Nucleic acid detection has become an essential feature in a wide range of analysis methods, including both methods for determining the presence or absence of a particular genetic element as well as methods for determining the identity of the particular genetic element. Determining the identity of variations in genetic elements among individuals is one example which has applications in genetic and infectious disease diagnosis, forensic techniques, tissue typing or monitoring genetic makeup in plants or animals in agriculture.

As most of the polymorphisms found in the human genome are single nucleotide polymorphisms (SNPs), genotyping methods will be important factors in the analysis of genetic variation caused by SNPs. Because SNPs underlie most of the known human genetic diseases, methods for rapidly and efficiently genotyping individuals have become essential in the search for disease-causing or predisposing genes. SNP analysis also plays an important role in drug design, as genetic variability in genes involved in drug metabolism pathways may significantly affect drug toxicity and efficacy. Genome wide linkage disequilibrium studies and association studies often require the genotyping of a large number of markers in certain sets of individuals, while diagnostic applications often require the genotyping of small numbers of markers in many individuals. Currently, most genotyping methods are not well suited for low cost, high throughput analysis, rendering many studies and diagnostic procedures prohibitively expensive or impossible

As a general overview, current methods for detecting SNPs include methods that discriminate among physical differences in nucleic acids, methods based on the recognition of nucleic acid mismatches by enzymes, methods based on ligase enzymes, allele-specific oligonucleotide probes (ASO) methods, and template directed primer extension methods.

However, even the most recently developed methods for detecting nucleic acids, and SNPs in particular, are not well suited to high throughput analysis due to tedious protocols, expensive reagents and lack of sensitivity. Currently available template directed primer extension methods, for example, typically require expensive enzymes, tedious size separation steps, or cumbersome enzymatic detection steps. Other methods, such as ASO probes, are inflexible as genotyping methods and lack sufficient specificity to be able to discriminate among many SNPs simultaneously, and require lengthy optimization times for each SNP to be detected.

In view of the disadvantages of current nucleic acid detection methods and in view of the

large numbers of analyses that need to be carried out, it is evident that there is a need for reagent-efficient, highly sensitive nucleic acid detection methods and systems which can be automated and used in high throughput formats.

SUMMARY OF THE INVENTION

The present invention is based on novel methods for detecting a target nucleotide in a nucleic acid sample. The invention provides nucleic acid detection methods and assay systems and kits based on nucleic acid template dependent primer extension assays having increased robustness, efficiency and throughput capacity. The methods of the present invention generally involve the use of nucleotides comprising a detectable label which are incorporated sequence-specifically into an oligonucleotide primer. The methods may be used for any application where a target nucleotide is to be detected, and are of particular use in genotyping for diagnostics and disease association studies.

In a first embodiment, a method for the detection of a target nucleotide in a nucleic acid sample comprises performing a microsequencing assay in the presence of a fluorescently labeled nucleotide, and detecting the incorporation of said nucleotide by measuring fluorescence intensity. In this embodiment, the method for the detection of a target nucleotide in a nucleic acid sample comprises (a) providing a nucleic acid sample comprising a target nucleic acid wherein the nucleotide bases spanning said target nucleic acid are unpaired; (b) contacting said sample with an oligonucleotide primer capable of hybridizing specifically to a stretch of nucleotides bases present in said sample 3' to said target nucleic acid;(c) subjecting a hybrid formed in step (b) to a single nucleotide template-dependent primer extension reaction in the presence of a chain terminating nucleotide comprising a fluorescent label, wherein said nucleotide is capable of being incorporated sequence specifically into the primer; and (d) detecting the incorporation of a labeled nucleotide in the primer by measuring direct fluorescence intensity.

Direct fluorescence intensity, preferably total fluorescence intensity, can be measured without the need to measure changes in polarization. In certain embodiments, incorporation of a nucleotide is detected by quantitatively measuring a change or decrease in direct fluorescence intensity, wherein said change or decrease indicates the incorporation of a labeled nucleotide into an oligonucleotide primer due to quenching of the dye label upon incorporation into an oligonucleotide. In other embodiments, background fluorescence due to unincorporated labeled nucleotides is eliminated, and incorporation of a nucleotide is detected by measuring direct fluorescence to determine either the substantial presence or absence of a signal.

In another embodiment of the invention, a method for the detection of a target nucleotide in a nucleic acid sample comprises performing a microsequencing assay, wherein oligonucleotide primers capable of hybridizing to both complementary strands of a nucleic acid sample 3' of said target nucleotide are provided in the same well. In this embodiment, the invention provides a method for the detection of a target nucleotide in a nucleic acid sample comprising (a) providing a

nucleic acid sample comprising a target nucleic acid, said nucleic acid sample having a first strand and a second strand complementary thereto, wherein the nucleotide bases spanning said target nucleic acid are unpaired; (b) contacting said sample with a first oligonucleotide primer capable of hybridizing specifically to a stretch of nucleotides bases present in said first strand of sample 3' to said target nucleic acid; (c) contacting said sample with a second oligonucleotide primer capable of hybridizing specifically to a stretch of nucleotides bases present in said second strand of sample 3' to said target nucleic acid; (d) subjecting a hybrid formed in steps (b) and (c) to a single nucleotide template-dependent primer extension reaction in the presence of at least two different chain terminating nucleotides, wherein each type of nucleotide comprises a distinct fluorescent label and is capable of being incorporated sequence specifically into the primer;(e) detecting the incorporation of a labeled nucleotide in the primer.

In another embodiment referred to as the PCR passivation method, the inventors have provided a means for the reduction of background signal in fluorescence based nucleic acid detection methods. The invention embodies a method for the detection of a target nucleotide in a nucleic acid sample comprising (a) Providing a nucleic acid sample comprising a target nucleic acid; (b) treating said nucleic acid sample with unlabeled nucleotides in the presence of a polymerase; and (c) conducting a template directed primer extension assay, thereby detecting a target nucleotide in said sample. In preferred embodiments of the PCR passivation method, the step of conducting a template directed primer extension assay comprises: (a) Providing a nucleic acid sample comprising a target nucleic acid; (b) contacting said sample with an oligonucleotide primer capable of hybridizing specifically to a stretch of nucleotides bases present in said sample; (c) subjecting a hybrid formed in step (b) to a nucleic acid template-dependent primer extension reaction in the presence of a nucleotide comprising a fluorescent label, wherein said nucleotide is capable of being incorporated sequence specifically into the primer; and (d) detecting the incorporation of a labeled nucleotide in the primer. Optionally, said oligonucleotide primer is capable of hybridizing 3' to the target nucleotide in said nucleic acid sample. Optionally, said primer extension reaction is a single nucleotide primer extension reaction. Optionally, the incorporation of said chain terminating nucleotide is detected by measuring fluorescence intensity. Optionally, the incorporation of said chain terminating nucleotide is detected by measuring fluorescence polarization. In another preferred embodiment of the PCR passivation method, the step of conducting a template directed primer extension assay comprises a) providing a nucleic acid sample comprising a target nucleic acid, said nucleic acid sample having a first strand and a second strand complementary thereto, wherein the nucleotide bases spanning said target nucleic acid are unpaired; (b) contacting said sample with a first oligonucleotide primer capable of hybridizing specifically to a stretch of nucleotides bases present in said first strand of sample 3' to said target nucleic acid; (c) contacting said sample with a second oligonucleotide primer capable of hybridizing specifically to a stretch of nucleotides bases present in said second strand of sample 3' to said target nucleic acid; (d) subjecting

a hybrid formed in steps (b) and (c) to a single nucleotide template-dependent primer extension reaction in the presence of at least two different chain terminating nucleotides, wherein each type of nucleotide comprises a distinct fluorescent label and is capable of being incorporated sequence specifically into the primer; and (e) detecting the incorporation of a labeled nucleotide in the primer.

Optionally, in any of the nucleic acid detection methods of the invention, the oligonucleotide primer is capable of hybridizing immediately 3' to the target nucleotide in the nucleic acid sample. Optionally, in any of the nucleic acid detection methods of the invention, the nucleic acid template-dependent primer extension reaction comprises temperature cycling. Optionally, in any of the nucleic acid detection methods of the invention, the nucleic acid template-dependent primer extension reaction is carried out in the absence of dATP, dTTP, dGTP or dCTP. Optionally, in any of the nucleic acid detection methods of the invention, said nucleotides comprise fluorescently labeled ddATP, ddTTP, ddGTP and ddCTP. Optionally, each type of chain terminating nucleotide comprises a distinct fluorescent label. Optionally, ddATP and ddTTP are labeled with a first fluorescent dye and ddCTP and ddGTP are labeled with a second fluorescent dye. Optionally, a first nucleotide selected from the group consisting of ddATP and ddTTP labeled with a first fluorescent dye, and a second nucleotide selected from the group consisting of ddCTP and ddGTP labeled with a second fluorescent dye. Furthermore, said fluorescently labeled nucleotides may be provided as a mixture.

Optionally, any of the nucleic acid detection methods of the invention comprises providing a fluorescent control molecule to the single nucleotide primer extension reaction, wherein the fluorescent dye is distinct from those used in the nucleic acid template dependent primer extension reaction, such that the fluorescent control molecule serves as an internal control for removal of unincorporated labeled ddNTPs from extended oligonucleotide primers.

Preferably, a nucleic acid sample comprises at least one single nucleotide polymorphism.

The nucleic acid detection methods of the invention further comprise separating unincorporated labeled nucleotides from oligonucleotide primers extended by said nucleic acid template-dependent primer extension reaction prior to detection of fluorescence.

The nucleic acid detection methods of the invention may further comprise spatially separating oligonucleotide primers which have been extended by the single nucleotide primer extension reaction. The nucleic acid detection methods of the invention may comprise attaching an oligonucleotide primer or a nucleic acid sample to a solid support. Preferably, a solid support comprises an addressable oligonucleotide array.

The invention further provides a method for the detection of a target nucleotide in a nucleic acid sample comprising: (a) performing a nucleic acid amplification reaction on a sample nucleic acid, (b) purifying said nucleic acid sample, and (c) detecting a target nucleotide according to any of the nucleic acid detection methods of the invention. Said purification step (b) of the nucleic acid sample preferably comprises removing amplification primers and dNTPs. Optionally, said

purification step comprises treating a nucleic acid sample with an enzyme.

The invention also provides method for determining the genotype of a selected organism at a genetic locus, detecting an association between a genotype and a trait, estimating the frequency of a haplotype for a set of biallelic markers in a population, and detecting an association between a haplotype and a trait. In one embodiment, the invention provides a method for determining the genotype of a selected organism at one or more genetic loci comprising: (a) obtaining from the organism a sample containing genomic DNA (b) identifying the target nucleotide bases present at each of the one or more polymorphic sites of interest according to a nucleic acid detection method of the invention, and (c) determining the genotype of said organism at one or more genetic loci based on the different alleles identified in step (b).

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows an overview of a direct fluorescence genotyping method in homogenous phase based on a microsequencing (MIS) protocol.

Figures 2A and 2B shows results from the genotyping of nucleic acid samples using the homogenous phase direct fluorescence method for 6 nucleic acid samples, normalized to the fluorescent intensity of controls.

Figures 3A and 3B each show a C/T polymorphism genotyped using the direct fluorescence detection microsequencing method with separation of unincorporated labeled ddNTP.

DETAILED DESCRIPTION

1. Nucleic acid detection method formats

A wide range of methods are currently used for determining the identity of a target nucleotide. Many genotyping methods require the previous amplification of the DNA region carrying the target nucleotide of interest. While the amplification of the nucleic acid sample prior to carrying out a nucleic acid detection protocol is often preferred at present, ultrasensitive detection methods which do not require amplification are also known.

A. Physical property differences

One class of nucleic acid detection methods relies on the detection of physical properties of a target DNA. Such genotyping methods for the detection of biallelic polymorphisms include single strand conformational polymorphism analysis (SSCP), denaturing gradient gel electrophoresis (DGGE) and denaturing high-performance liquid chromatography (dHPLC). SSCP has been described by Orita et al.(1989); DGGE has been described by Fisher et al., (1983) and dHPLC-based methods have been described by Liu et al. (1997/1998). These detection methods are generally

carried out following amplification of a DNA sample by an amplification method such as PCR. These methods can be used to identify homozygotes and heterozygotes, but do not provide information on the genotype.

B. RFLP

Restriction Fragment Length Polymorphisms (RFLP) are among the first generations of genetic markers used in genotyping studies, and involve the detection of polymorphisms which are in a recognition sequence for a restriction endonuclease. However, RFLP-based methods are relatively time consuming and costly, and RFLP markers occur at lower frequency and uniformity than SNPs as a whole, meaning that it will be more difficult to find an RFLP marker near a genetic locus of interest.

C. Mismatch recognition

Other nucleic acid detection methods are based on enzymatic mismatch recognition. Variations can be carried out using a mismatch-recognizing endonuclease, or using an mismatch-recognizing enzyme which binds but does not cleave the sample nucleic acid. Several examples using different enzymes are described in International Patent Publication WO93/20233. In one exemplary method, a mismatch repair enzymes from the mutS family of enzymes (Modrich (1991)) binds to a mismatch and, when the sample is treated with an exonuclease, prevents digestion of the locus of the mismatch. The presence of a protected nucleic acid indicates the presence of a mutation. (International Patent Publication No WO 95/29258).

D. Ligation based methods

Ligation based methods depend on particularly stringent requirements for correct base pairing of the 3' end of an amplification primer to a target DNA sequence and the joining by a ligase of two oligonucleotides hybridized to a target DNA sequence; these methods are thus sensitive to mismatches close to a ligation site, especially at the 3' end. For example, the "Oligonucleotide Ligation Assay" (OLA) uses two oligonucleotides which are designed to be capable of hybridizing to abutting sequences of a single strand of a target molecules. One of the oligonucleotides is biotinylated, and the other is detectably labeled. If the precise complementary sequence is found in a target molecule, the oligonucleotides will hybridize such that their termini abut, and create a ligation substrate that can be captured and detected. OLA is capable of detecting single nucleotide polymorphisms and may be advantageously combined with PCR as described further below. The OLA assay, however, requires complicated label detection steps, as well as steps for capturing, separating and washing the oligonucleotides.

E. Allele specific hybridization probes

For high throughput genotyping, methods based on allele specific oligonucleotide (ASO) probes have been used extensively. ASO probe based methods, particularly using probes or nucleic acid samples immobilized on a solid support, are used in an assay format which can be automated. Furthermore, labeling and detection method are relatively inexpensive. Either the probe or the PCR

product which is the nucleic acid sample may be labeled. However, ASO probe methods have low specificity and require at the very least two probes per target nucleotide to be detected. ASO probes must be optimized to discriminate single nucleotide mismatches, which can be time consuming when a large number of probes must be used. Furthermore, probe specificity can be difficult to obtain in some cases. In practice, each ASO probe requires optimization; in some cases, to diminish optimization time, up to 40 probes in parallel are used to detect a single target nucleotide. Attempts have been made to reduce error rates by detecting and averaging results using a large number of variations of hybridization probes varying in sequences adjacent to the polymorphic site. Thus, the ASO probe method lacks flexibility and specificity for use in genotyping for most SNP association studies.

F. Template Directed Primer Extension Methods

Template directed primer extension assays rely on a template dependent polymerase enzyme to sequence specifically extend a nucleic acid primer which is hybridized to a nucleic acid template. Allele specificity of template directed primer extension methods may be derived from the use of allele specific primers as well as from the allele specific incorporation of nucleotides into extended primers. Known primer extension methods include DNA sequencing, allele-specific PCR, single oligonucleotide primer extension and microsequencing methods.

(i) DNA Sequencing:

Of the template-directed primer extension based methods, electrophoresis based DNA sequencing involving both size and color separation is the most definitive and reliable. However, DNA sequencing is expensive both in terms of reagents and time required to gather and interpret the sequence information. For this reason, other polymerase based methods such as allele specific PCR and microsequencing methods are more suitable to high throughput environments.

(ii) Allele Specific Amplification

Other template-directed primer extension based methods particularly suited for the detection of single nucleotide polymorphism include amplification methods such as PCR. In general, allele specific PCR assays involve the use of a labeled oligonucleotide primer capable of discriminating between two alleles of a target nucleic acid, such that an amplification product can be generated and detected only if an allele of interest is present.

(iii) Microsequencing assays

Template-directed primer extension based methods for the detection of nucleic acids include microsequencing assays, wherein an oligonucleotide primer is extended by a single nucleotide complementary to the nucleotide to be detected. Herein, microsequencing methods are also referred to as template-directed single nucleotide primer extension methods. In microsequencing methods, a nucleic acid sample which serves as the template is treated, if such nucleic acid is double-stranded, so as to obtain unpaired nucleotide bases spanning the target nucleotide position. An oligonucleotide primer is provided to hybridize to the nucleic acid sample 3' to a target nucleotide to

be detected. Preferably, the oligonucleotide primer is provided to hybridize to the nucleic acid sample immediately 3' to the target nucleotide. Optionally, the oligonucleotide primer may hybridize several nucleotides removed 3' to the target nucleotide, as long as the sequence in the nucleic acid sample between the 3' end of the primer and the target oligonucleotide do not contain a nucleotide of the same type as the target nucleotide to be detected. The primer-template hybrid is subjected to a primer extension reaction in the presence of labeled dideoxynucleoside triphosphates (ddNTP), whereby the 3' end of the primer is extended by a single nucleotide. The identity of the incorporated labeled ddNTP is then determined by one of several label detection methods. Microsequencing may be used interchangably with single nucleotide template dependent primer extension. An example of a microsequencing assay is described in European Patent No. 0 412 883 B1, the disclosure of which is incorporated herein by reference.

As an example, microsequencing reactions are often carried out using fluorescent ddNTPs, and the extended microsequencing primers are analyzed by electrophoresis on ABI 377 sequencing machines to determine the identity of the incorporated nucleotide. Alternatively, capillary electrophoresis are used in order to process a higher number of assays simultaneously.

G. Detection Methods

Many systems for detecting labeled nucleotides are currently available. Combinations of labels, detection methods and assay formats for carrying out detection of a label include the use of electrophoresis, mass spectrometry, enzymatic and fluorescent labeling based assays, as described below in Table 1.

Table 1: Detection methods and detection formats

Label Detection Method	Label measurement	Detection Format
With size separation:		
Electrophoresis device	Fluorescence	Slab gel, capillaries, microfluidic
Mass spectrometry		chips
Without size separation:		
Enzymatic based assay	Fluorescence	Microtiter plate,
	Colorimetry	Microtiter plate
Fluorescent labeling (blot)	Direct fluorescence	Microtiter plate, chip, membrane
	FRET	Microtiter plate

Polarized fluorescence Microtiter plate

While each method has certain advantages and disadvantages, most currently available methods have not been suitable for high throughput applications. For example, electrophoresis separation takes a long time to conduct relative to mass spectrometry and other detection methods without size separation. Mass spectrometry, however, is a serial process which tends to be unsuitable for high throughput environments as parallel detectors are not currently available. Furthermore, mass spectrometry requires the absence of Na+ salts in the solution which implies a solid phase desalting process for every genotyping reaction. In summary mass spectrometry produces few false genotyping results, but is sensitive to assay conditions. ELISA-based assays are time consuming, reagent consuming, and not well suited to automation as they typically require two wells per sample which double the number of assay points, or require a cascade of different ELISA reactions, several washes and/or incubation with two different substrates. Other methods for detection of a label involve detecting fluorescent dyes. Methods based on fluorescent dyes, however, have typically been poorly suited to high throughput environments and/or have had sensitivity disadvantages. Fluorescence resonance energy transfer (FRET) based label detection involves the use of two fluorophores such that emission spectrum of one overlaps the excitation spectrum of another. Thus, in methods such as Kwok et al., US Patent 5,945,283 and Kwok (1997), a signal may be detected from a primer containing a first fluorophore when a second fluorophore is incorporated by a primer extension reaction. While FRET has advantages in that it is well adapted to formats where samples are in unpurified form, FRET requires time consuming probe development and expensive probe labeling. Detection using polarized fluorescence can differentiate between single stranded and hybridized double stranded nucleic acids without physical separation of the two forms, and is thus well adapted to formats where samples are in unpurified form (see Murakami, et al. (1991), European Patent Publication No. 0 382 433, PCT Patent Publication No. WO 92/18650 and Chen et al., (1999)). However, fluorescence polarisation has not been validated as a detection method for genotyping purposes and is not currently suitable for a high throughput environment due to lack of available apparatus capable of detecting incorporated labels with sufficient sensitivity.

While each having certain disadvantages for high throughput applications, some of the different label and detection means discussed above have nevertheless been incorporated into several genotyping typing systems. Several such examples include Syvanen et al (1990, 1992), Hall and Smirnoff (1997), Braun (1997), Sokolov (1990) Kuppuswami (1991), Ugozzoli (1992), Nikiforov (1993), Shumaker (1996) and Pecheniuk (1997).

2. Direct Fluorescence Detection Methods of the Invention

Currently available template directed primer extension assay methods suffer to various extents from lack of sensitivity and throughput capacity; for example, methods based on

measurement of fluorescence polarization have not been suitable for use in a high throughput environment because available detection systems and devices are not capable of providing the required sensitivity. The present method therefore provides a method suitable for a high throughput environment by allowing detection using currently available detection apparatus.

The inventors provide a novel template directed primer extension-based nucleic acid detection method carried out in the presence of a fluorescently labeled nucleotide, wherein the incorporation of a labeled nucleotide is detected by directly measuring fluorescence intensity. As used herein, direct detection refers to the measurement of signal intensity at a given wavelength, without the necessity to measure other characteristics of a signal such as changes in the polarization of fluorescence. Thus, in preferred embodiments, total fluorescence intensity is measured, and a single intensity measurement at a given wavelength can allow detection of an incorporated labeled nucleotide.

In preferred embodiments described further below, primer extension methods based on direct fluorescence are carried out in formats where a separation step allows unincorporated labeled nucleotides to be removed prior to the detection step (e.g. by filtration or degradation) as well as in homogenous phase formats where no further separation of unincorporated nucleotides is carried out.

Microsequencing-Based Direct Fluorescence Detection Methods

(a) Direct fluorescence detection in homogenous phase

The quantum yield of fluorescent dyes depends strongly on their close environment (e.g., pH and polarity of the solvent, pKs of functional groups on the dye molecules; for instance, it is well known that pKs of amino-acids in a peptide or a protein molecule depend on neighboring amino-acids and are different than pKs of free amino acids). The quenching of fluorescent dyes attached to an oligonucleotide upon hybridization has been described by Cantor and collaborators more than 20 years ago (Koenig, P. at al (1977); Talavera, E.M. (1997); and Seidel C. et al. (1991)).

Based on the concept that the quantum yield of a free, fluorescently-labeled ddNTP will be different than the quantum yield of a dye incorporated into an oligonucleotide, and even more different than the quantum yield of an oligonucleotide/PCR amplicon hybrid, the present inventors have provided a novel nucleic acid detection method which can be carried out in a homogenous phase (eg single tube, or does not require separation of unincorporated labeled oligonucleotides) and which does not require that the polarization of fluorescence be measured.

Due to fluorescence quenching, the fluorescence of a labeled ddNTP decreases when it is incorporated into an oligonucleotide and hybridizes onto a complementary nucleic acid strand, such as that of a PCR amplicon. As shown in Figure 1, a microsequencing oligonucleotide primer is brought into contact with a nucleic acid sample template (PCR amplicon) and labeled ddNTP. A microsequencing (MIS) reaction is carried out such that a labeled ddNTP is incorporated into said

oligonucleotide primer in a template-directed, sequence-specific manner. By detecting a decrease in fluorescence intensity after the microsequencing reaction, the identity of the incorporated ddNTP can be determined. The decrease in fluorescent intensity allows genotyping to be performed by measuring direct fluorescence without any purification to separate labeled unincorporated ddNTPs.

The homogenous phase direct fluorescence detection method of the invention involves first providing a nucleic acid sample which comprises a target nucleotide. The nucleic acid sample is treated, if such nucleic acid is double-stranded, so as to obtain unpaired nucleotide bases spanning the target nucleotide position. In preferred embodiments, the single nucleotide primer extension reaction is carried out using temperature cycling according to PCR methods involving repeating cycles of denaturation, hybridization and extension temperatures, such that the nucleic acid sample is denatured. If a nucleic acid sample as provided to the primer extension reaction is single-stranded, this denaturation step is not necessary.

The nucleic acid sample is contacted with an oligonucleotide primer capable of hybridizing specifically to a stretch of nucleotides in the sample 3' to the target nucleotide to be detected; preferably the primer hybridizes immediately 3' to the target nucleotide. The sample and the primer form a hybrid, which is subjected to a template-dependent single nucleotide primer extension reaction.

The primer extension reaction mixture will contain, in addition to said hybrid, a template dependent enzyme capable of extending the primer in a sequence specific manner, and a chain terminating nucleotide comprising a fluorescent label. Any suitable template-dependent enzyme capable of sequence specifically extending the primer can be used, as further described below; an exemplary polymerase which can be used is ThermoSequenase (Amersham, E79000G). With respect to nucleotides, at least two different ddNTPs, each comprising a distinct fluorescent label are provided. The primer extension reaction is preferably carried out in the absence of dNTPs. Any suitable fluorescent dye may be used, examples of which are provided herein; the examples shown herein used R110 and TAMRA. Generally, any fluorescent dye that may change quantum yield upon changes in the local environment can be used. As shown in examples further described below, incorporation of R110-ddNTP into an oligonucleotide and consequent hybridization of the labeled oligonucleotide onto a complementary DNA strand yields in relatively strong quenching of the R110 fluorescent dye, in contrast to e.g. Tamra-ddNTP for which the quenching is somewhat less profound. Assuming that the fluorescence quenching is a result of change in local environment of the dye (i.e., from polar aqueous medium to non-polar environment in close proximity to the complementary DNA strand) it is expected that the quenching will be stronger for polar dyes such as for example R110, NAN, FAM, HEX, TET, ZOE. Combinations of different ddNTPs and fluorescent labels can be provided in any suitable combination and mixture. For example, for detection using four colors, four ddNTPs (ddATP, ddTTP, ddCTP and ddGTP) each labeled with a distinct label are provided. In another example, two color detection is used, wherein two ddNTPs

are provided, each labeled with a distinct fluorescent dye. Optionally, two color detection can also involve providing four different ddNTPs labeled with two distinct fluorescent dyes. Preferably, two color detection is used and one color is used for ddATP and ddTTP, and another color for ddCTP and ddGTP, offering a wider range of available detection apparati and allowing simple adjustment of detection conditions on said apparatus. The single nucleotide primer extension reaction is carried out according to a protocol suitable for the template dependent enzyme; preferably temperature is cycled, such as in PCR protocols.

A single chain terminating nucleotide is thereby incorporated into a primer only if said chain terminating nucleotide is complementary to the target nucleotide. Detection of labeled ddNTPs incorporated into a primer is detected by measuring a change or decrease in direct fluorescence intensity, indicating that all or a portion of the ddNTPs labeled with a particular dye have been incorporated into the oligonucleotide primer. The change in fluorescence intensity can be measured in any suitable manner. For example, fluorescence intensity can be measured upon addition of labeled ddNTP to the primer extension reaction mixture but before the primer extension reaction has taken place, and after the primer extension reaction. Change in fluorescence intensity can also be measure after the primer extension reaction in a test sample and in a control sample. In another example, change in fluorescence intensity can be measured vis-à-vis an internal control dye; one or more additional fluorescently labeled nucleotides or fluorescent small molecules, wherein the fluorescent dye is distinct from those used in the primer extension reaction, can be provided to the primer extension reaction mixture. Direct fluorescence intensity can be measured as less than total fluorescence intensity (eg as one polarization), but is preferably measured as the total fluorescence intensity.

Thus, by measuring total fluorescence intensity, the invention allows a user to avoid performing multiple fluorescence measurements at different polarizations.

Additionally, one or more additional fluorescently labeled nucleotides or fluorescent small molecules, wherein the fluorescent dye is distinct from those used to label nucleotides capable of being incorporated in the oligonucleotide primer, is provided as an internal control standardizing the measurements of decreases in fluorescence intensity.

As described further herein, the direct detection method of the invention can be carried out using any of a wide range of suitable nucleic acid samples, oligonucleotide primers, fluorescent dye, template dependent enzymes, and detection devices. The direct detection method of the invention may be carried out in any suitable assay format. In one embodiment the direct detection method of the invention is carried out in a format in which extended primers are purified, and wherein microtiter plates are used; microtiter plates are well suited to high throughput environment and may be used conveniently with detection apparati developed for standardized plate format. The methods of the invention can also be carried out in an assay format wherein at least one step is conducted on a solid phase. In a solid phase, an oligonucleotide primer or a sample nucleic acid is attached to a

solid support. The methods of the invention may also be carried out on an array or chip, wherein an oligonucleotide primer or a sample nucleic are spatially separated. Methods for attaching nucleic acids to a solid support are well known to those of skill in the art; further examples are described herein.

The present inventors further provide a method for nucleic acid detection wherein amplification of a nucleic acid sample and detection of a nucleic acid are carried out in a single well. In one aspect, the method thus comprises (a) amplifying a nucleic acid sample; (b) purifying a nucleic acid sample; and (c) detecting a nucleic acid according to the homogenous phase direct detection method of the invention. Said purification step generally involves removing amplification primers and dNTPs, and can be carried out prior to performing a single nucleotide primer extension reaction, or prior to the addition of labeled nucleotides to the primer extension reaction mixture. A preferred purification means for comprises treating said amplification product with at least one nuclease enzyme; preferably Shrimp Alkaline Phosphatase is used. In a preferred embodiment, the enzyme used for removal of amplification primers and dNTPs is inactivated by temperature treatment; preferably the enzyme is inactivated by temperature cycling carried out for single nucleotide primer extension in the direct detection method of the invention.

The direct detection method of the invention can also be carried out on an integrated system which may, for example, combine any of the detection formats of the invention with other reactions or processes. In one embodiment, an integrated system comprises means for (a) amplifying a nucleic acid sample and (b) detecting a nucleic acid according to the direct detection method of the invention. In another aspect, an integrated system comprises (a) amplifying a nucleic acid sample; (b) purifying a nucleic acid sample; and (c) detecting a nucleic acid according to the homogenous phase direct detection method of the invention.

A protocol used to carry out a homogenous phase detection assay for genotyping is disclosed in Example 2.

Figures 2A and 2B shows results from the genotyping of nucleic acid samples using the homogenous phase direct fluorescence method for 6 samples. The dyes TAMRA and R110 were used to label two different ddNTPs. Samples 1 to 4 represent homozygotes, samples 1 and 2 being homozygous for a first base and samples 3 and 4 homozygous for a second base. Samples 5 and 6 were heterozygotes.

Figure 2A shows controls for the measurement of decreases in fluorescence intensity; fluorescence intensity was measured using the dyes TAMRA and R110 in the absence of the oligonucleotide primer (T-ol) and in the absence of nucleic acid sample (T-DNA). For each of 8 controls, the T-oligo is shown in the first column and the T-DNA is shown in the second column from the right. Figure 2B shows fluoresence intensity for sample 1 to 6 ; on the "y" axis is plotted the reversed (total) fluorescent intensity normalized to the intensity of T-ol. Based on the controls, a threshold value of about 1.1, once greater and 1.0, is set. Figures 2A and 2B thus demonstrate that

genotyping from microsequencing reactions can be done in a homogeneous phase (without purification) by direct fluorescence reading, thereby simplifiing instrumentation requirements.

(b) Direct fluorescence detection with removal of unincorporated label

The identity of a labeled nucleotide incorporated into a microsequencing oligonucleotide can also be determined using direct fluorescence without the need to measure the quenching of fluorescence. By removing unincorporated labeled nucleotides, background fluorescence can be decreased such that only total intensity as an indication of substantial presence or absence of signal need be measured. While adding a step of removing (e.g. by filtration, degradation) unincorporated ddNTPs, this method allows for highly robust genotyping without the need for complicated detection devices.

The direct fluorescence detection method of the invention involves first providing a nucleic acid sample which comprises a target nucleotide. The nucleic acid sample is treated, if such nucleic acid is double-stranded, so as to obtain unpaired nucleotide bases spanning the target nucleotide position. In preferred embodiments, the single nucleotide primer extension reaction is carried out using temperature cycling according to PCR methods involving repeating cycles of denaturation, hybridization and extension temperatures, such that the nucleic acid sample is denatured. If a nucleic acid sample as provided to the primer extension reaction is single-stranded, this denaturation step is not necessary.

The nucleic acid sample is contacted with an oligonucleotide primer capable of hybridizing specifically to a stretch of nucleotides in the sample 3' to the target nucleotide to be detected; preferably the primer hybridizes immediately 3' to the target nucleotide. The sample and the primer form a hybrid, which is subjected to a template-dependent single nucleotide primer extension reaction.

The primer extension reaction mixture will contain, in addition to said hybrid, a template dependent enzyme capable of extending the primer in a sequence specific manner, and a chain terminating nucleotide comprising a fluorescent label. Any suitable template-dependent enzyme capable of sequence specifically extending the primer can be used, as further described below; an exemplary polymerase which can be used is ThermoSequenase (Amersham, E79000G). With respect to nucleotides, at least two ddNTPs, each comprising a distinct fluorescent label are provided. The primer extension reaction is preferably carried out in the absence of dNTPs. Any suitable fluorescent dye may be used, examples of which are provided herein; preferred examples include FAM and/or TAMRA.

Combinations of different ddNTPs and fluorescent labels can be providcd in any suitable combination and mixture. For example, for detection using four colors, four ddNTPs (ddATP, ddTTP, ddCTP and ddGTP) each labeled with a distinct label are provided. In another example, two color detection is used, wherein two ddNTPs are provided, each labeled with a distinct fluorescent

dye. Optionally, two color detection can also involve providing four different ddNTPs labeled with two distinct fluorescent dyes. Preferably, two color detection is used and one color is used for ddATP and ddTTP, and another color for ddCTP and ddGTP, offering a wider range of available detection apparati and allowing simple adjustment of detection conditions on said apparati.

The single nucleotide primer extension reaction is carried out according to a protocol suitable for the template dependent enzyme; preferably temperature is cycled, such as in PCR protocols.

Prior to detection of incorporated labeled ddNTPs in extended oligonucleotide primers, unincorporated labeled ddNTPs are removed in order to reduce non-specific background fluorescence. Any suitable method for removal of ddNTPs may be used; in an exemplary method, a G50 gel (Sephadex) is used in a microtiter plate format.

Additionally, one or more additional fluorescently labeled nucleotides or fluorescent small molecules, wherein the fluorescent dye is distinct from those used to label nucleotides capable of being incorporated in the oligonucleotide primer, is provided as an internal control for removal of unincorporated labeled ddNTPs. For example, a labeled nucleotide may be used as a control and added subsequent to carrying out a single nucleotide primer extension reaction.

A single chain terminating nucleotide is thereby incorporated into a primer only if said nucleotide is complementary to the target nucleotide. Detection of labeled ddNTPs incorporated into a primer may be detected by measuring fluorescence intensity using any suitable detection instrument. The invention advantageously allows detection to be performed by measuring total fluorescence intensity, without the need to perform multiple measurements at different polarizations. A preferred instrument is the Fluorimager (Molecular Dynamics) or Analyst (LJL Biosystems).

As described further herein, the direct detection method of the invention can be carried out using any of a wide range of suitable nucleic acid samples, oligonucleotide primers, fluorescent dye, template dependent enzymes, and detection devices. Furthermore, as described herein, the direct detection method of the invention may also be used with a variety of suitable assay formats.

Optionally, the nucleic acid sample is capped by the addition of an unlabeled terminator to the 3' end of the nucleic acid sample before performing the primer extension reaction. A terminator such as a ddNTP is capable of terminating a template-dependent, primer extension reaction such that no additional labeled terminator will attach at the 3' end of the nucleic acid sample.

Figures 3A and 3B each show a C/T polymorphism genotyped using the direct fluorescence detection microsequencing method with separation of unincorporated labeled ddNTP, using one microsequencing oligonucleotide primer, a PCR passivation step, and a TAMRA-ddCTP and FAM-ddUTP incorporation protocol. The reaction was measured on the Analyst (LJL Biosystems). Shown in Figure 3A and 3B are the raw fluorescent data in the FAM and TAMRA channel. HMZ1 are C/C homozygotes, HMZ2 are T/T homozygotes, HTZ are C/T heterozygotes, T-Oligo is a negative control without the microsequencing oligonucleotide primer, and T-DNA is a negative control

without PCR product.

This direct detection method of the invention may be carried out in any suitable assay format. In particular, the methods of the invention may be carried out in formats where the primers extended by the reaction for the incorporation of a labeled nucleotide are purified, or where said primer remain in unpurified form. In one embodiment, a purification step is carried out on a solid phase.

In one example of a format where extended primers are purified, the direct detection method of the present invention is conducted as described above. Four ddNTPs, each labeled with a distinct fluorescent dye are provided to the single nucleotide primer extension reaction mixture. Prior to detection of incorporated labeled ddNTPs in primers, a purification step to remove unincorporated labeled ddNTPs is carried out in order to reduce non-specific background fluorescence. As an example, purification may be carried out on a Sephadex G50 gel, which is suitable for use with microtiter plates. Incorporation of labeled ddNTPs is carried out by measuring fluorescence intensity. An example of a suitable detection device is the Fluorimager (Molecular Dynamics) or Analyst (LJL Biosystems).

Another example of a format where extended primers are purified involves conducting the direct detection method of the present invention as described herein in a two color detection format. In this format, the single nucleotide primer extension reaction is performed in the presence of two ddNTPs corresponding to two possible alleles present at the target nucleic acid, each ddNTP labeled with a distinct fluorescent dye. Oligonucleotide primers are designed to hybridize immediately 3' to a target nucleotide to be detected. One or more additional fluorescently labeled nucleotides or fluorescent small molecules is provided as an internal control for removal of unincorporated labeled ddNTPs. The fluorescent dye of said control molecule is distinct from those used in the primer extension reaction. Prior to detection of incorporated labeled ddNTPs in primers, a purification step to remove unincorporated labeled ddNTPs is carried out in order to reduce non-specific background fluorescence. As an example, purification may be carried out on a Sephadex G50 gel, which furthermore is suitable for use in microtiter plate format. Incorporation of labeled ddNTPs is detected by measuring fluorescence intensity. Purification is checked by verifying that no fluorescence from the internal control for removal of unincorporated labeled ddNTPs remains.

In a most preferred example of a format where extended primers are purified, the direct detection method of the present invention is conducted as a microsequencing assay as described herein, in a two color detection format. In this preferred method, a sample nucleic acid is treated with unlabeled ddNTPs in the presence of a polymerase as further described in the "PCR passivation" method herein in section 4. The PCR passivation method fills in nicks and elongated unfinished nucleic acid fragments, thereby reducing background fluorescence. The sample may be subjected to a purification step after the PCR passivation step. The single nucleotide primer extension reaction is then performed in the presence of two ddNTPs, each labeled with a distinct

fluorescent dye. One or more additional fluorescently labeled nucleotides or fluorescent small molecules is provided as an internal control for removal of unincorporated labeled ddNTPs. The fluorescent dye of said control molecule is distinct from those used in the primer extension reaction. Prior to detection of incorporated labeled ddNTPs in primers, a purification step to remove unincorporated labeled ddNTPs is carried out in order to reduce non-specific background fluorescence. As an example, purification may be carried out on a Sephadex G50 gel, which furthermore is suitable for use in microtiter plate format. Incorporation of labeled ddNTPs is detected by measuring fluorescence intensity. Purification is checked by verifying that no fluorescence from the internal control for removal of unincorporated labeled ddNTPs remains.

In one embodiment the direct detection method of the invention is carried out in a format in which extended primers are purified, and wherein microtiter plates are used; microtiter plates are well suited to high throughput environment and may be used conveniently with detection apparati developed for standardized plate format.

The methods of the invention can also be carried out in an assay format wherein at least one step is conducted on a solid phase. In a solid phase, an oligonucleotide primer or a sample nucleic acid is attached to a solid support. The methods of the invention may also be carried out on an array or chip, wherein an oligonucleotide primer or a sample nucleic are spatially separated. Methods for attaching nucleic acids to a solid support are well known to those of skill in the art; further examples are described herein.

The present inventors further provide a method for nucleic acid detection wherein amplification of a nucleic acid sample and detection of a nucleic acid are carried out in a single well. In one aspect, the method thus comprises (a) amplifying a nucleic acid sample; (b) purifying a nucleic acid sample; and (c) detecting a nucleic acid according to the direct detection method of the invention. Said purification step generally involves removing amplification primers and dNTPs, and can be carried out prior to performing a single nucleotide primer extension reaction, or prior to the addition of labeled nucleotides to the primer extension reaction mixture. A preferred purification means for comprises treating said amplification product with at least one nuclease enzyme; preferably Shrimp Alkaline Phosphatase is used. In a preferred embodiment, the enzyme used for removal of amplification primers and dNTPs is inactivated by temperature treatment; preferably the enzyme is inactivated by temperature cycling carried out for single nucleotide primer extension in the direct detection method of the invention.

The direct detection method of the invention can also be carried out on an integrated system which may, for example, combine any of the detection formats of the invention with other reactions or processes. In one embodiment, an integrated system comprises means for (a) amplifying a nucleic acid sample and (b) detecting a nucleic acid according to the direct detection method of the invention. In another aspect, an integrated system comprises (a) amplifying a nucleic acid sample; (b) purifying a nucleic acid sample; and (c) detecting a nucleic acid according to the direct detection

method of the invention.

3. Double Oligonucleotide Method

Currently, template-based single nucleotide primer extension assays suffer from highly variable hybridization yield from one oligonucleotide primer to another, resulting in highly variable signal intensity from one primer extension assay to another. This can be a problem in a high throughput environment where a single standard protocol must be used.

In a high throughput microsequencing genotyping system developed by the present inventors, a validation or optimization step is usually necessary in order to identify oligonucleotide primers which give low or no signals. Prior to use in microsequencing assays, these oligonucleotides are then eliminated from use in assays, and new oligonucleotides designed to bind to the opposite strand of a sample nucleic acid can be prepared for testing in another assay. However, this additional testing step slows the nucleic acid detection process and decreases throughput of the system considerably.

Thus, in order to increase the throughput and robustness of this and other methods of nucleic acid detection methods, the present inventors provide a method for reducing oligonucleotide primer validation turn around time and for reducing signal intensity variation in primer extension assays. In the present method, a target nucleotide is detected on both of the two complementary strands of a nucleic acid sample in a single assay, thereby diminishing hybridization yield variation and increasing assay sensitivity. This method is referred to herein as the double oligonucleotide method.

Microsequencing Assays

The present double oligonucleotide method is most preferably carried out as a microsequencing based method, involving first providing a nucleic acid sample comprising a target nucleotide to be detected. The nucleic acid sample is treated, if such nucleic acid is double-stranded, so as to obtain unpaired nucleotide bases spanning the target nucleotide position. In preferred embodiments, where the single nucleotide primer extension reaction is carried out using temperature cycling according to PCR methods involving repeating cycles of denaturation, hybridization and extension temperatures such that the nucleic acid sample is denatured. If a nucleic acid sample as provided to the primer extension reaction is single-stranded, this denaturation step is not necessary.

Oligonucleotide primers are provided such that a first oligonucleotide primer is designed to hybridize immediately 3' to a target nucleotide on a first strand of a nucleic acid sample, and a second oligonucleotide primer is designed to hybridize immediately 3' to a target nucleotide on a second complementary strand of the nucleic acid sample.

A hybrid formed by the sample and the primer is subjected to a single nucleotide template-dependent primer extension reaction in the presence of a template-dependent enzyme and a chain terminating nucleotide. Any suitable template-dependent enzyme capable of sequence specifically

extending the primer can be used, as further described below; an exemplary polymerase which can be used is ThermoSequenase (Amersham, E79000G).

The primer extension reaction is carried out in the presence of fluorescently labeled ddNTPs and preferably in the absence of dNTPs. Combinations of different ddNTPs and labels can be provided in any suitable combination or mixture. For example, in a four-color detection method, four ddNTPs (ddATP, ddTTP, ddCTP and ddGTP) each labeled with a distinct fluorescent label are provided. In another example, two color detection is used, wherein the four ddNTPs are labeled with two distinct fluorescent dyes; one color is used for ddATP and ddTTP, and another color for ddCTP and ddGTP. This two color, four ddNTP mixture can detect all SNPs except A/T and G/C polymorphisms which represent only 15% of all SNPs on average.

Two color detection is particularly preferred because it is suitable for a wide range of detection apparati and particularly suitable for high-throughput analysis. However, while the present method in a four color detection format allows detection of all types of nucleotide polymorphisms, the two color detection format with four ddNTPs does not allow the detection of A/T and G/C polymorphism types in certain embodiments. Nevertheless, two color, four ddNTP detection is a preferred format because A/T and G/C polymorphisms together constitute on average only about 15% of human polymorphisms. In embodiments involving spatial resolution of primer extension products such as chip-type solid supports, all polymorphism types may be detected using a two color format.

Any suitable fluorescent dye may be used; dyes are described further herein. Suitable dyes, for example, are FAM and/or TAMRA.

Prior to detection of incorporated labeled ddNTPs in primers, a purification step to remove unincorporated labeled ddNTPs is carried out in order to reduce non-specific background fluorescence. As an example purification may be carried out on a Sephadex G50 filter, which furthermore is suitable for use in microtiter plate format.

Optionally, one or more fluorescently labeled nucleotides or fluorescent small molecules comprising a fluorescent dye distinct from those used in the primer extension reaction may be provided as an internal control for removal of unincorporated labeled ddNTPs. In one embodiment, a labeled nucleotide is used as a control and is added subsequent to carrying out the single nucleotide primer extension reaction.

Any detection method suitable for detection of a fluorescent label may be used to detect the labeled nucleotide incorporated into the oligonucleotide primer. Exemplary detection methods comprise direct measurement of fluorescence intensity, measurement of fluorescence polarization and fluorescence resonance energy transfer (FRET). If a FRET based method is used for detection of an incorporated nucleotide, a fluorescently labeled oligonucleotide primer can be used.

Assay formats

The double oligonucleotide method of the invention may be carried out in any suitable assay format. In particular, the methods of the invention may be carried out in formats where the primers extended by the reaction for the incorporation of a labeled nucleotide are purified, where said primers remain unpurified. In one embodiment, a purification step is carried out on a solid phase.

In one example, a format in which extended primers are purified comprises conducting the double oligonucleotide method of the present invention in a microsequencing format as described herein. Oligonucleotide primers are provided such that a first oligonucleotide primer is capable of hybridizing immediately 3' to a target nucleotide on a first strand of a nucleic acid sample, and a second oligonucleotide primer is capable of hybridizing immediately 3' to a target nucleotide on a second, complementary strand of a nucleic acid sample. Different ddNTPs, each labeled with a distinct fluorescent dyes are provided to the single nucleotide primer extension reaction. Incorporation of a fluorescently labeled ddNTP is then detected by any suitable characteristic and means.

In another example, a format in which extended primers are purified comprises conducting the two oligonucleotide primer detection method of the present invention in a microsequencing format. Oligonucleotide primers are provided such that a first oligonucleotide primer is capable of hybridizing to a nucleic acid sample immediately 3' to a target nucleotide on a first strand of the nucleic acid sample, and a second oligonucleotide primer is capable of hybridizing to a nucleic acid sample immediately 3' to a target nucleotide on a second complementary strand of the nucleic acid sample. Four ddNTPs, labeled with two distinct fluorescent dyes are provided, such that ddATP and ddTTP are labeled with a first dye and ddCTP and ddGTP are labeled with a second dye are provided to the single nucleotide primer extension reaction. Incorporation of a fluorescently labeled ddNTP is then detected by any suitable characteristic and means, including but not limited to the measurement of fluorescence intensity, fluorescence polarization or fluorescence resonance energy transfer.

In a further and particularly preferred example, a format in which extended primers are purified comprises conducting the double oligonucleotide detection method of the present invention in a microsequencing format as described herein. Oligonucleotide primers are provided such that a first oligonucleotide primer is capable of hybridizing to a nucleic acid sample immediately 3' to a target nucleotide on a first strand of the nucleic acid sample, and a second oligonucleotide primer is capable of hybridizing to a nucleic acid sample immediately 3' to a target nucleotide on a second complementary strand of the nucleic acid sample. The single nucleotide primer extension reaction is carried out in the presence of four ddNTPs, labeled with two distinct fluorescent dyes, such that ddATP and ddTTP are labeled with a first dye, and ddCTP and ddGTP are labeled with a second dye. One or more additional fluorescently labeled nucleotides or fluorescent small molecules comprising a fluorescent dye distinct from those used in the single nucleotide primer extension reaction are provided as an internal control for removal of unincorporated labeled ddNTPs. Prior to

detection of incorporated labeled ddNTPs in primers, a purification step to remove unincorporated labeled ddNTPs is carried out in order to reduce non-specific background fluorescence. As an example, purification may be carried out on a Sephadex G50 gel, particularly for use in a microtiter plate format. Incorporation of labeled ddNTPs is carried out by measuring the fluorescence intensity of the dyes used to label ddNTPs. Purification is checked by verifying that no fluorescence from the internal control for removal of unincorporated labeled ddNTPs remains. An example of a suitable detection device is the Fluorimager (Molecular Dynamics) or Analyst (LJL Biosystems).

In one embodiment, the double oligonucleotide method of the invention is carried out in a format in which extended primers are purified, and wherein microtiter plates are used; microtiter plates are well suited to high throughput environment and may be used conveniently with detection apparati developed for standardized plate format.

The methods of the invention can also be carried out in an assay format wherein at least one step is conducted on a solid phase. In a solid phase, an oligonucleotide primer or a sample nucleic acid is attached to a solid support. Methods of the invention may also be carried out on an array or chip, wherein nucleic acids (i.e. an oligonucleotide primer or a sample nucleic acid) are spatially separated. Methods for attaching nucleic acids to a solid support are well known to those of skill in the art; further examples are described herein.

In one embodiment, the direct detection method of the invention is carried out in a purified format wherein microtiter plates are used; microtiter plates are well suited to high throughput environment and may be used conveniently with detection apparati developed for standardized plate format.

The methods of the invention can also be carried out in an assay format wherein at least one step is conducted on a solid phase. In a solid phase, an oligonucleotide primer or a sample nucleic acid is attached to a solid support. The methods of the invention may also be carried out on an array or chip, wherein an oligonucleotide primer or a sample nucleic are spatially separated. Methods for attaching nucleic acids to a solid support are well known to those of skill in the art; further examples are described herein.

The present inventors further provide a method for nucleic acid detection wherein amplification of a nucleic acid sample and detection of a nucleic acid are carried out in a single well. In one aspect, the method thus comprises (a) amplifying a nucleic acid sample; (b) purifying a nucleic acid sample; and (c) detecting a nucleic acid according to the double oligonucleotide method of the invention. Said purification step generally involves removing amplification primers and dNTPs, and can be carried out prior to performing a single nucleotide primer extension reaction, or prior to the addition of labeled nucleotides to the primer extension reaction mixture. A preferred means for comprises treating said amplification product with at least one nuclease enzyme; preferably Shrimp Alkaline Phosphatase is used. In a preferred embodiment, the enzyme used for removal of amplification primers and dNTPs is inactivated by temperature treatment; preferably the

enzyme is inactivated by temperature cycling carried out for single nucleotide primer extension in the direct detection method of the invention.

The double oligonucleotide method of the invention can also be carried out on an integrated system which may, for example, combine any of the detection formats of the invention with other reactions or processes. In one embodiment, an integrated system comprises means for (a) amplifying a nucleic acid sample and (b) detecting a nucleic acid according to the double oligonucleotide method of the invention. In another aspect, an integrated system comprises (a) amplifying a nucleic acid sample; (b) purifying a nucleic acid sample; and (c) detecting a nucleic acid according to the double oligonucleotide method of the invention.

4. **PCR Passivation Method**

Template directed primer extension based nucleic methods which do not discriminate based on size generally suffer from high non specific background signal. The present inventors provide a method for the detection of a nucleic acid which reduces background signal caused by non-specific incorporation of labeled nucleotides into a nucleic acid sample during a primer extension reaction. The present invention is based on the principle that fluorescently labeled ddNTPs are incorporated in nucleic acid samples at nicks or unfinished elongated fragments by a polymerase which is used in the primer extension step of a nucleic acid detection method. This non-specified incorporation is of particular concern where a nucleic acid sample has been subjected to an amplification reaction. This background level of fluorescence is very difficult to monitor since the incorporation of fluorescent ddNTPs into the nucleic acid sample is in competition with the specific primer extension and thus depends strongly on hybridization rate of the oligonucleotide primers to the nucleic acid sample.

In order to avoid such artifacts, the inventors have developed a method which provides a template-dependent primer extension-based method for detecting a nucleic acid comprising a step of treating a nucleic acid sample with unlabeled nucleotides in the presence of a polymerase prior to the primer extension reaction, thereby filling in nicks in the nucleic acid sample. The method is referred to herein as the "PCR passivation" method, in view of the particular advantage when used with nucleic acid samples that have been previously amplified. However, the present method is not limited to use with samples prepared by PCR methods or amplification methods in general, and may be used with any nucleic acid sample of any origin and regardless of how said nucleic acid sample was treated. The PCR passivation method is particularly advantageous when only spectral separation detection modes are used, such as the measurement of fluorescence intensity (direct fluorescence detection) and fluorescence polarization. Essentially any nucleotide may be used for treatment of the nucleic acid sample so long as the analog does not contain a label capable of causing substantial background signal when used with a particular detection instrument or method. Generally, all four native nucleotide bases are used for treatment of the nucleic acid sample.

While the PCR passivation method may be used in any suitable nucleic acid detection method, the PCR passivation method is particularly useful in microsequencing based assays. Such assays involve (a) incorporating unlabeled ddNTPs into a nucleic acid sample in order to fill in the nicks according to the methods of the invention, and (b) conducting a single nucleotide primer extension reaction. In preferred embodiments, the PCR passivation method is used for microsequencing based methods with direct fluorescence or polarized fluorescence-based detection methods.

The PCR passivation method may further be used in combination with the double primer method of and the direct fluorescence method described herein.

Assay formats

The PCR passivation method may be used in any suitable nucleic acid detection method, and moreover in any suitable microsequencing-based method. In particular, the methods of the invention may be carried out in formats where the primer extended by the reaction for the incorporation of a labeled nucleotide are purified, or where said primers are in unpurified form. A purification step can be carried out on a solid phase. Notably, the PCR passivation methods have been found to be well suited for use in unpurified or purified format methods using microtiter plates. Preferably, a nucleic acid sample is a double stranded nucleic acid.

In a first example of a nucleic acid detection method comprising PCR passivation and carried out in a format where extended primers are purified, a nucleic acid sample is treated with unlabeled ddNTPs in order to fill in nicks in a nucleic acid sample prior to conducting a single nucleotide primer extension reaction. Treatment with unlabeled ddNTPs is carried out in the presence of a template dependent enzyme such as the polymerases described herein. Reaction conditions for filling in nicks with unlabeled nucleotides, preferably ddNTPs, can be carried out according to the polymerase manufacturer's instructions, or according to methods described herein. Following treatment with unlabeled ddNTPs, the primer extension reaction is carried out in the presence of four ddNTPs, each labeled with a distinct fluorescent dye. One or more additional fluorescently labeled nucleotides or fluorescent small molecules are also provided as an internal control for removal of unincorporated labeled ddNTPs. The fluorescent dye of said internal control is distinct from those used in the single nucleotide primer extension reaction. Prior to detection of incorporated labeled ddNTPs in primers, a purification step to remove unincorporated labeled ddNTPs is carried out in order to reduce non-specific background fluorescence. Purification may be carried out on a Sephadex G50 gel. Detection of incorporated labeled ddNTPs is carried out by measuring fluorescence intensity. An example of a suitable detection device is the Fluorimager (Molecular Dynamics) or Analyst (LJL Biosystems).

In another example of a nucleic acid detection method comprising the PCR passivation method and carried out in a format where extended primers are purified, a nucleic acid sample is treated with unlabeled ddNTPs in order to fill in nicks in a nucleic acid sample as described above

prior to conducting a primer extension reaction. The primer extension reaction is carried out in the presence of two ddNTPs, each labeled with a distinct fluorescent dye. One or more additional ddNTP labeled with a fluorescent dye distinct from those used in the primer extension reaction are provided as an internal control for removal of unincorporated labeled ddNTPs. Prior to detection of incorporated labeled ddNTPs in primers, a purification step to remove unincorporated labeled ddNTPs is carried out in order to reduce non-specific background fluorescence. Purification may be carried out on a Sephadex G50 filter. Detection of incorporated labeled ddNTPs is carried out by measuring fluorescence intensity. An example of a suitable detection device is the Fluorimager (Molecular Dynamics) or Analyst (LJL Biosystems).

In a further, most preferred example of a nucleic acid detection method comprising PCR passivation, a nucleic acid sample is treated with unlabeled ddNTPs in order to fill in nicks in a nucleic acid sample, as described above, prior to conducting a single nucleotide primer extension reaction. The single nucleotide primer extension reaction is carried out in the presence of four ddNTPs, each labeled with a distinct fluorescent dye. One or more additional ddNTPs labeled with a fluorescent dye distinct from those used in the primer extension reaction are also provided as an internal control for removal of unincorporated labeled ddNTPs. Prior to detection of incorporated labeled ddNTPs in primers, a purification step to remove unincorporated labeled ddNTPs is carried out in order to reduce non-specific background fluorescence. Purification may be carried out on a Sephadex G50 filter. Detection of incorporated labeled ddNTPs is carried out by measuring fluorescence intensity. An example of a suitable detection device is the Fluorimager (Molecular Dynamics) or Analyst (LJL Biosystems)

The methods of the invention can also be carried out in an assay format wherein at least one step is conducted on a solid phase. In a solid phase, an oligonucleotide primer or a sample nucleic acid is attached to a solid support. A solid support is generally an array or chip, wherein oligonucleotide primers or a sample nucleic are spatially separated. Said arrays or chips may be addressable. Methods for attaching nucleic acids to a solid support are well known to those of skill in the art; further examples are described herein.

In one embodiment, the direct detection method of the invention is carried out in a purified format wherein microtiter plates are used; microtiter plates are well suited to high throughput environment and may be used conveniently with detection apparati developed for standardized plate format.

The present inventors further provide a method for nucleic acid detection wherein amplification of a nucleic acid sample and detection of a nucleic acid are carried out in a single well. In one aspect, the method thus comprises (a) amplifying a nucleic acid sample; (b) purifying a nucleic acid sample; (c) treating said nucleic acid with unlabeled nucleotides, preferably ddNTPs, in the presence of a polymerase enzyme, and (d) detecting a nucleic acid. Said purification step generally involves removing amplification primers and dNTPs, and can be carried out prior to

performing a single nucleotide primer extension reaction, or prior to the addition of labeled nucleotides to the primer extension reaction mixture. A preferred means for carrying our said purification step of (b) comprises treating said amplification product with at least one nuclease enzyme; preferably Shrimp Alkaline Phosphatase is used. In a preferred embodiment, the enzyme used for removal of amplification primers and dNTPs is inactivated by temperature treatment; preferably the enzyme is inactivated by temperature cycling carried out for single nucleotide primer extension in the direct detection method of the invention.

The PCR passivation method of the invention can also be carried out on an integrated system which may, for example, combine any of the detection formats of the invention with other reactions or processes. In one aspect, an integrated system comprises (a) amplifying a nucleic acid sample and (b) treating said nucleic acid according to the PCR passivation method of the invention. In another aspect, an integrated system comprises (a) purifying a nucleic acid sample, (b) treating said nucleic acid according to the PCR passivation method of the invention, and (c) detecting a nucleic acid; preferably said step of detecting a nucleic acid is comprises conducting a microsequencing assay; optionally, said step of detecting a nucleic acid is performed according to the double oligonucleotide method of the invention. Said purification step may involve removing amplification primers and dNTPs. Means for removal of amplification primers and dNTPs may include gels, filters and enzymatic treatment.

5. Solid Supports

Any of the primers or nucleic acid samples in the nucleic acid detection methods described herein can be attached to a solid support using means known in the art.

Any of the primers or nucleic acid samples in the nucleic acid detection methods described herein can be labeled, if desired, by incorporating a label detectable by spectroscopic, photochemical, biochemical, immunochemical, or chemical means. Preferably, primers are labeled at their 3' and 5' ends. A label can be used to capture the primer or nucleic acid sample, so as to facilitate the immobilization of either the primer or a primer extension product, such as amplified DNA, on a solid support. A capture label is attached to the primer or nucleic acid sample and can be a specific binding member which forms a binding pair with the solid phase's reagent's specific binding member (e.g. biotin and streptavidin).

6. Oligonucleotide Arrays

Oligonucleotides attached to a solid support may comprise an array of oligonucleotides. An oligonucleotide primer or nucleic acid sample may be attached to a solid support in a high-density format.

The nucleic acid detection methods of the invention may also be carried out in high density oligonucleotide array format. Pastinen et al.(1997), for example describes a method for multiplex detection of single nucleotide polymorphism in which a solid phase minisequencing method is

applied to an oligonucleotide array format. High-density arrays of DNA probes attached to a solid support (DNA chips) are further described below.

Any polynucleotide provided herein may be attached in overlapping areas or at random locations on the solid support. Alternatively, the polynucleotides of the invention may be attached in an ordered array wherein each polynucleotide is attached to a distinct region of the solid support which does not overlap with the attachment site of any other polynucleotide. Preferably, such an ordered array of polynucleotides is designed to be "addressable" where the distinct locations are recorded and can be accessed as part of an assay procedure. Addressable polynucleotide arrays typically comprise a plurality of different oligonucleotide probes that are coupled to a surface of a substrate in different known locations. The knowledge of the precise location of each polynucleotides location makes these "addressable" arrays particularly useful in hybridization assays. Any addressable array technology known in the art can be employed with the polynucleotides of the invention. In general, methods for preparing oligonucleotide arrays can involve the chemical coupling of 5'-modified primers as miniaturized spots on an activated glass surface using high capacity printing robots (Lamture et al, 1994 and Schena et al, 1996). Arrays produced by this method have achieved spots of 100 μm in diameter and spacing of 500μm.

7. Nucleic acid detection kits

The present invention also provides reagents and kits suitable for carrying out the nucleic acid detection methods of the invention. Kits are typically packaged to aid laboratories to carry out a particular procedure, and typically provide at least the reagents specially adapted to carrying out the particular procedure.

Thus, in one aspect of the invention, the present invention encompasses kits for the detection of nucleic acids according to the direct fluorescence detection method of the invention. Such kits may comprise oligonucleotide primers capable of hybridizing to a nucleic acid sample 3' to each allele of the target nucleic acid to be detected, and a nucleotide labeled with a fluorescent label, wherein the fluorescent label is suitable for use in detection methods based on the measurement of direct fluorescence intensity. Preferably, at least two different nucleotides are provided, each labeled with a distinct fluorescent dye. In one aspect, the dyes may be dyes that change quantum yield upon changes in the local envirnonment, e.g. result in a quenching, or a decrease in fluorescence intensity when incorporated into an oligonucleotide primer. In one embodiment, four different nucleotides are provided, each labeled with a distinct fluorescent dye; in another embodiment, four different nucleotides are provided, labeled with two distinct fluorescent dyes. Optionally, the kit includes a further fluorescent dye, as a labeled dNTP, ddNTP or small fluorescent molecule for example, as a control for the removal of unincorporated labeled ddNTPs after the single nucleotide primer extension reaction. In one example, a kit may comprise the dyes TAM or FAMRA.

In another aspect of the invention, the present invention encompasses kits for the detection of nucleic acids according to the double primer method of the invention. Such kits may comprise oligonucleotide primers capable of hybridizing to a nucleic acid sample 3' to each allele of the target nucleic acid to be detected, and two ddNTPs, each labeled with a distinct fluorescent dye. In one embodiment, four ddNTPs are provided, each ddNTP labeled with a distinct fluorescent dye.

In any kit of the invention, the oligonucleotide primers and/or the labeled ddNTPs may be provided as a mixture. Any kit of the invention may further optionally comprise a polymerase; optionally nuclease enzymes are provided to remove amplification primers or dNTPs; optionally gel filters are provided to remove amplification primers, dNTPs or unincorporated fluorescently labeled nucleotides. Labeled nucleotides may be provided to the primer extension reaction mixture in separate steps, or as a mixture of nucleotides. Preferred kits of the invention comprise ddNTPs labeled with FAM and TAMRA dyes.

Optionally, a kit is provided such to permit detection of a large number of nucleic acid targets according to any nucleic acid detection method of the invention. In one embodiment, said kit may provide a reaction substrate such as a microtiter plate. In a preferred embodiment, said microtiter plate is suitable for use in a purified or unpurified format assay. Optionally, a purification means adapted to said microtiter plate is provided for purified format assay kits.

The present invention comprises the use of any of the nucleic acid detection kits of the invention for genotyping. Preferably one or more SNPs are genotyped; optionally at least 10, 100, 1000, 10000 or 100000 different SNPs are genotyped in at least 1, 10, 100, 1000 or 10000 individuals.

8. Methods Of Genetic Analysis Using The Methods Of The Present Invention

While the present methods may be used in any nucleic acid detection application, genotyping applications for diagnostics and disease association or pharmacogenomic studies are of particular interest. Various methods for interpreting results from genotyping assays are known in the art, examples of which are further described in copending US patent application no. 09/326,402, filed June 4, 1999 and International Patent Publication No. WO 99/65590, the contents of both of which are hereby incorporated in their entireties. Methods for generating a high-density map of biallelic markers have been described in US Patent Application filed January 14, 2000 titled "Biallelic markers for use in constructing a high density disequilibrium map of the human genome" and PCT Publication No. WO 99/04038. A set of biallelic polymorphisms that could be used as genetic markers has been described in WO 98/20165 and in copending US Patent Application nos. 09/298,850 and 09/422,978, filed April 21, 1999 October 20, 1999 respectively, the disclosures of which are incorporated herein by reference.

A method is provided for determining the genotype of a selected organism at one or more genetic loci comprising (a) obtaining from the organism a sample containing genomic DNA and (b) identifying the nucleotide bases present at each of the one or more target polymorphic sites

according to a nucleic acid detection method of the invention, and (c) determining the genotype of said organism at one or more genetic loci based on the different alleles identified in step (b).

In one aspect, the nucleic acid detection methods of the present invention are used in diagnostic methods capable of identifying individuals who express a detectable trait as the result of a specific genotype or individuals whose genotype places them at risk of developing a detectable trait at a subsequent time. The present diagnostics may be used to diagnose any detectable trait, including any disease, predisposition to disease, age of onset of a detectable symptom, drug response, drug efficacy, treatment response, treatment efficacy and drug toxicity. Such a diagnosis can be useful in the monitoring, prognosis and/or prophylactic or curative therapy.

In another aspect, several different methods are available for the genetic analysis of complex traits (Lander and Schork, 1994). The search for disease-susceptibility genes can be conducted a linkage approach (Weir, 1996; Morton, 1955; Ott, 1991) including parametric or nonparametric methods, or an association approach (Khoury et al., 1993). Statistical methods and computer programs useful for linkage analysis are well-known to those skilled in the art (Terwilliger J.D. and Ott J., 1994; Ott J., 1991).

Association studies explore the relationships among frequencies for sets of alleles between loci, and may involve approaches such as genome-wide association studies, candidate region association studies and candidate gene association studies. Allele frequencies or haplotype frequencies are used in association studies. Allele frequencies are determined directly from results obtained from the genotyping methods of the present invention. When the gametic phase is not known, haplotype frequencies can be estimated from the multilocus genotypic data. Any method known to person skilled in the art can be used to estimate haplotype frequencies (Lange K., 1997; Weir, B.S., 1996) Preferably, maximum-likelihood haplotype frequencies are computed using an Expectation- Maximization (EM) algorithm (see Dempster et al., 1977; Excoffier L. and Slatkin M., 1995). Haplotype estimations are usually performed by applying the EM algorithm using for example the EM-HAPLO program (Hawley M. E. et al., 1994) or the Arlequin program (Schneider et al., 1997).

The present invention thus relates to a method of estimating the frequency of an allele of a biallelic marker in a population comprising: (a) genotyping individuals from said population for said biallelic marker according to a nucleic acid detection method of the invention; and (b) determining the proportional representation of said biallelic marker in said population.

Further encompassed by the present invention is a method of detecting an association between a genotype and a trait, comprising the steps of (a) determining the frequency of at least one biallelic marker in trait positive population; (b) determining the frequency of at least one biallelic marker in a control; and (c) determining whether a statistically significant association exists between said genotype and said trait, wherein at least one of said steps for determining the frequency of a biallelic marker comprises detecting a target nucleotide according to a nucleic acid detection method

of the invention.

Additionally, included is a method of estimating the frequency of a haplotype for a set of biallelic markers in a population, comprising: (a) genotyping at least one biallelic marker for each individual in said population; (b) genotyping a second biallelic marker by determining the identity of the nucleotides at said second biallelic marker for both copies of said second biallelic marker present in the genome of each individual in said population; and c) applying a haplotype determination method to the identities of the nucleotides determined in steps a) and b) to obtain an estimate of said frequency, wherein at least one of said steps of genotyping at least one biallelic marker comprises detecting a target nucleotide according to a nucleic acid detection method of the invention.

Preferably, said haplotype determination method is selected from the group consisting of asymmetric PCR amplification, double PCR amplification of specific alleles, the Clark algorithm, or an expectation-maximization algorithm. Further provided is a method of detecting an association between a haplotype and a trait, comprising the steps of (a) estimating the frequency of at least one haplotype in a trait positive population according to the method of estimating the frequency of a haplotype above; (b) estimating the frequency of said haplotype in a control population according to said method of estimating the frequency of a haplotype above; and (c) determining whether a statistically significant association exists between said haplotype and said trait.

9. Nucleic Acid Samples

The nucleic acid sample containing a target nucleotide to be detected can be from any source. The sample of nucleic acids can be natural or synthetic (i.e., synthesized enzymatically in vitro). The sample of nucleic acids can comprise deoxyribonucleic acids, ribonucleic acids, or copolymers of deoxyribonucleic acid and ribonucleic acid. The nucleic acid of interest can be synthesized enzymatically in vivo, synthesized enzymatically in vitro, or synthesized nonenzymatically. The sample containing the nucleic acid or acids of interest can comprise genomic DNA from an organism, RNA transcripts thereof, or cDNA prepared from RNA transcripts thereof. The sample containing the nucleic acid or acids of interest can also comprise extragenomic DNA from an organism, RNA transcripts thereof, or cDNA prepared from RNA transcripts thereof. Also, the nucleic acid or acids of interest can be synthesized by the polymerase chain reaction.

These nucleic acid samples can be obtained from sources including human and animal body fluids such as whole blood, serum, plasma, cerebrospinal fluid, urine, lymph fluids, and various external secretions of the respiratory, intestinal and genitourinary tracts, tears, saliva, milk, white blood cells, myelomas and the like; biological fluids such as cell culture supernatants; fixed tissue specimens including tumor and non-tumor tissue and lymph node tissues; bone marrow aspirates and fixed cell specimens. For example, human genomic DNA can be obtained from peripheral venous blood of each donor. Techniques to prepare genomic DNA from biological samples are well known to the skilled technician, further described in Example 1.

The nucleic acid of interest can comprise one or more moieties that permit affinity separation of the nucleic acid of interest from the unincorporated reagent and/or the primer. The nucleic acid of interest can comprise biotin which permits affinity separation of the nucleic acid of interest from the unincorporated reagent and/or the primer via binding of the biotin to streptavidin which is attached to a solid support. The sequence of the nucleic acid of interest can comprise a DNA sequence that permits affinity separation of the nucleic acid of interest from the unincorporated reagent and/or the primer via base pairing to a complementary sequence present in a nucleic acid attached to a solid support. The nucleic acid of interest can be labeled with a detectable marker; this detectable marker can be different from any detectable marker attached to a primer or a nucleotide.

10. Amplification methods

Nucleic acid amplification techniques are well known to those skilled in the art. Examples of amplification techniques that can be used, particularly to amplify a nucleic acid sample to be detected according to the method of the invention include, the ligase chain reaction (LCR) described in EP-A- 320 308, WO 9320227 and EP-A-439 182, the polymerase chain reaction (PCR, RT-PCR) and techniques such as the nucleic acid sequence based amplification (NASBA) described in Guatelli J.C., et al.(1990) and in Compton J.(1991), Q-beta amplification as described in European Patent Application No 4544610, strand displacement amplification as described in Walker et al.(1996) and EP A 684 315 and, target mediated amplification as described in PCT Publication WO 9322461.

LCR and Gap LCR are exponential amplification techniques, both depend on DNA ligase to join adjacent primers annealed to a DNA molecule. In Ligase Chain Reaction (LCR), probe pairs are used which include two primary (first and second) and two secondary (third and fourth) probes, all of which are employed in molar excess to target. The first probe hybridizes to a first segment of the target strand and the second probe hybridizes to a second segment of the target strand, the first and second segments being contiguous so that the primary probes abut one another in 5' phosphate-3'hydroxyl relationship, and so that a ligase can covalently fuse or ligate the two probes into a fused product. In addition, a third (secondary) probe can hybridize to a portion of the first probe and a fourth (secondary) probe can hybridize to a portion of the second probe in a similar abutting fashion. Of course, if the target is initially double stranded, the secondary probes also will hybridize to the target complement in the first instance. Once the ligated strand of primary probes is separated from the target strand, it will hybridize with the third and fourth probes, which can be ligated to form a complementary, secondary ligated product. It is important to realize that the ligated products are functionally equivalent to either the target or its complement. By repeated cycles of hybridization and ligation, amplification of the target sequence is achieved. A method for multiplex LCR has also been described (WO 9320227), incorporated herein by reference. Gap LCR (GLCR) is a version of

LCR where the probes are not adjacent but are separated by 2 to 3 bases.

For amplification of mRNAs, it is within the scope of the present invention to reverse transcribe mRNA into cDNA followed by polymerase chain reaction (RT-PCR); or, to use a single enzyme for both steps as described in U.S. Patent No. 5,322,770 or, to use Asymmetric Gap LCR (RT-AGLCR) as described by Marshall et al.(1994). AGLCR is a modification of GLCR that allows the amplification of RNA.

The PCR technology is the preferred amplification technique used in the present invention. A variety of PCR techniques are familiar to those skilled in the art. For a review of PCR technology, see White (1997) and the publication entitled "PCR Methods and Applications" (1991, Cold Spring Harbor Laboratory Press). In each of these PCR procedures, PCR primers on either side of the nucleic acid sequences to be amplified are added to a suitably prepared nucleic acid sample along with dNTPs and a thermostable polymerase such as Taq polymerase, Pfu polymerase, or Vent polymerase. The nucleic acid in the sample is denatured and the PCR primers are specifically hybridized to complementary nucleic acid sequences in the sample. The hybridized primers are extended. Thereafter, another cycle of denaturation, hybridization, and extension is initiated. The cycles are repeated multiple times to produce an amplified fragment containing the nucleic acid sequence between the primer sites. PCR has further been described in several patents including U.S. Patents 4,683,195; 4,683,202; and 4,965,188.

11. Template Dependent Enzyme

Preferably, the template-dependent enzyme described herein is a DNA or RNA polymerase. Preferably, DNA polymerases without proofreading activity are used to avoid 3' to 5' degradation of the primer. Any suitable polymerase which is both template- and primer-dependent can be used; examples of suitable polymerases include a T7 DNA polymerase such as ThermoSequenase (Amersham, E79000G), T4 DNA polymerase, T3 RNA polymerase, T7 RNA polymerase, T. aquaticus DNA polymerase, E. coli DNA polymerase I or the Klenow fragment thereof, or a reverse transcriptase such as a retroviral reverse transcriptase. Reaction conditions and temperatures generally vary according to the polymerase used.

12. Purification of amplification product

As described herein, enzymatic purification is a preferred method of removing amplification primers and nucleotides from a nucleic acid sample which has been subjected to nucleic acid amplification. The enzymatic purification method as described in the examples herein comprises treatment of amplified nucleic acid sample with Shrimp Alkaline Phosphatase and Exonuclease I in Shrimp Alkaline Phosphatase buffer. Shrimp Alkaline Phosphatase can be replaced by other alkaline phosphatase such calf intestine derived phosphatase. Alternatively, such enzymatic purification methods can be replaced by a gel filtration using G50, G100, Sephacryl, or any other suitable sieving

matrix. Other means for purification of an amplification product include performing a solid phase extraction using streptavidin beads or any suitable solid support with a biotinylated oligonucleotide primer. Alternatively, any other suitable high affinity recognition systems may be used, such as antibody-based systems recognizing haptens such as digoxygenine, fluoresceine, cholesterol. Yet further alternatives for purification of amplified nucleic acid samples includes filtration, dialysis, or any size-based separation methods.

13. Oligonucleotide primers

The oligonucleotides primers of the present invention are able to form a hybrid structure with a nucleic acid sample containing the specific target nucleotide, due to complementarity with a portion of the nucleic acid sequence.

While the specificity of the probe for binding the target nucleotide sequence is generally maximized with 100% nucleotide homology, this is not essential; any lower degree of homology may be chosen, as long as the primer binds to the target with the desired specificity. In a preferred embodiment of the invention, primers are chosen to be at least 70%, at least 80%, at least 90% or at least 95% homologous to the target nucleotide sequence.

While any suitable primer length may be used, the length of oligonucleotide primers typically range from 8, 10, 15, 20, or 30 to 100 nucleotides, preferably from 8 to 50, more preferably from 15 to 30 nucleotides. Shorter primers tend to lack specificity for a target nucleic acid sequence and generally require cooler temperatures to form sufficiently stable hybrid complexes with the template. Longer probes and primers are expensive to produce and can sometimes self-hybridize to form hairpin structures. The appropriate length for primers and probes under a particular set of assay conditions may be empirically determined by one of skill in the art.

Specific hybridization or specific binding with respect to a polynucleotide to a complementary polynucleotide as used herein is intended to mean the formation of hybrids between a polynucleotide and a particular target polynucleotide sequence wherein the polynucleotide preferentially hybridizes to the target polynucleotide sequence over sequences other than the target polynucleotide. The formation of stable hybrids depends on the melting temperature (Tm) of the DNA. The Tm depends on the length of the primer or probe, the ionic strength of the solution and the G+C content. The higher the G+C content of the primer or probe, the higher is the melting temperature because G:C pairs are held by three H bonds whereas A:T pairs have only two. The GC content in the probes of the invention usually ranges between 10 and 75 %, preferably between 35 and 60 %, and more preferably between 40 and 55 %. These factors are described further in, for example, Sambrook et al. (Sambrook et al., Molecular Cloning: A Laboratory Manual 2nd Ed., 1989 Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.)

It will be appreciated that any primer having a 3' end immediately adjacent to the polymorphic nucleotide may be used as a microsequencing primer.

The primers and probes can be prepared by any suitable method, including, for example, cloning and restriction of appropriate sequences and direct chemical synthesis by a method such as the phosphodiester method of Narang et al.(1979), the phosphodiester method of Brown et al.(1979), the diethylphosphoramidite method of Beaucage et al.(1981) and the solid support method described in EP 0 707 592. The oligonucleotide or polynucleotide includes linear oligomers of natural or modified monomers including deoxyribonucleotides, ribonucleotides and the like, capable of specifically binding to a target polynucleotide.

14. Labels and fluorescent dyes

As described herein, the methods of the present invention involve the use of nucleotides (or nucleotide analogs) comprising a detectable label. Unless otherwise specified, detectable labels used herein may comprise any detectable labels as long as they can be spectrally distinguished. In certain embodiments of the invention, detection of incorporated nucleotide can also be performed using radioactive ddNTP (P32, P33, S35) or phosphorescence dyes, colorimetric ddNTP or chemiluminescent ddNTPs. Such dyes allow the incorporated form of the nucleotide to be separated from the un-incorporated form.

In certain embodiments the two different nucleotides used in the detection method are labeled with a distinct fluorescent dye. Commercially available fluorescently labeled ddNTPs suitable for use include by way of example and not limitation, ddNTP-FAM, ddNTP-JOE, ddNTP-TAMRA, ddNTP-ROX, ddNTP-TET, ddNTP-HEX, dyes of the Cy family, BODIPY ddNTP, chlororhodamine ddNTP, the Big Dyes ddNTP, the ET-Terminators ddNTP. Derivatives of coumarine, fluoresceine, rhodamine, and Texas Red, for example, may also be used as fluorescent dyes to label a nucleotide.

Further details and examples for suitable fluorescent dyes are available in Pesce et al,. eds, Fluorescence Spectroscopy, Marcel Dekker, New York, 1971; White et al., Fluorescence Analysis: A practical Approach, Marcel Dekker, New York, 1970; Handbook of Fluorescent Probes and Research Chemicals, 6th Ed, Molecular Probes, Inc., Eugene, Oreg., 1996, the disclosures of which are incorporated herein by reference).

15. Purification for ddNTP removal

Unincorporated labeled nucleotides may be removed using any suitable means. In particular, a gel filtration method using gels such as the Sephadex G50 gel may be used. Other means for purification of an amplification product include performing a solid phase extraction using streptavidin beads or any suitable solid support with a biotinylated oligonucleotide primer. Alternatively, any other suitable high affinity recognition systems may be used, such as antibody-based systems recognizing haptens such as digoxygenine, fluoresceine, cholesterol. Further alternatives for purification of amplified nucleic acid samples includes filtration, dialysis, or size-

based separation methods.

Throughout this application, various publications, patents and published patent applications are cited. The disclosures of all of these publications, patents and published patent specification referenced in this application are hereby incorporated by reference into the present disclosure to more fully describe the state of the art to which this invention pertains.

EXAMPLES

Example 1

Direct Fluorescence Genotyping

Described in examples below are genotyping reactions according to the methods of the present invention. The examples below include one item of each sections below in the following order: sample preparation, PCR, PCR purification, microsequencing, removal of the dyes, fluorescence detection. Each subsection provides alternative protocols for the respective step of the genotyping reaction.

1 Sample preparation

30 ml of peripheral venous blood are taken from each donor in the presence of EDTA. Cells (pellet) are collected after centrifugation for 10 minutes at 2000 rpm. Red cells are lysed by a lysis solution (50 ml final volume: 10 mM Tris pH7.6; 5 mM $MgCl_2$; 10 mM NaCl). The solution is centrifuged (10 minutes, 2000 rpm) as many times as necessary to eliminate the residual red cells present in the supernatant, after resuspension of the pellet in the lysis solution.

The pellet of white cells is lysed overnight at 42°C with 3.7 ml of lysis solution composed of:

- 3 ml TE 10-2 (Tris-HCl 10 mM, EDTA 2 mM) / NaCl 0 4 M
- 200 µl SDS 10%
- 500 µl K-proteinase (2 mg K-proteinase in TE 10-2 / NaCl 0.4 M).

For the extraction of proteins, 1 ml saturated NaCl (6M) (1/3.5 v/v) is added. After vigorous agitation, the solution is centrifuged for 20 minutes at 10000 rpm.

For the precipitation of DNA, 2 to 3 volumes of 100% ethanol are added to the previous supernatant, and the solution is centrifuged for 30 minutes at 2000 rpm. The DNA solution is rinsed three times with 70% ethanol to eliminate salts, and centrifuged for 20 minutes at 2000 rpm. The pellet is dried at 37°C, and resuspended in 1 ml TE 10-1 or 1 ml water. The DNA concentration is evaluated by measuring the OD at 260 nm (1 unit OD = 50 µg/ml DNA).

To determine the presence of proteins in the DNA solution, the OD 260 / OD 280 ratio is determined. DNA preparations having a OD 260 / OD 280 ratio between 1.8 and 2 can be used in the subsequent examples described below.

2 PCR: PCR conditions

Amplification of the nucleic acid sample was done in a microtiter plate format (Thermofast,

Advance Biotech, Polylabo n° 35 355) in a Tetrad PTC-225 thermal cycler (MJ Research). Human Genomic DNA (25 ng) from each individual was amplified in a 25 µl reaction mixture containing 10 mM Tris-Hcl (pH 8.4), 50mM KCl, 0.3 µM of each PCR primer (17-22 specific mers, GENSET, crude synthesis, 10 OD), 0.1 mM dNTP (Boehringer), 2mM MgCl2 (Perkin-Elmer) and 0.5 unit of AmpliTaq GOLD DNA polymerase (Perkin-Elmer). Thermal cycling conditions were done by an initial denaturation step at 94°C for 10 min., followed by 35 cycles at 94° for 30 sec, 55°C for 60 sec and 72°C for 30 sec. Once finished, the cycles were followed by an elongation step at 72° for 7 min..

3 PCR Purification: Enzymatic PCR purification step

5 µl of PCR products were added to 5 µl purification mixture containing Shrimp Alkaline Phosphatase (2U, Amersham E70092X), Exonuclease I (2U, Amersham E70073Z) in Shrimp Alkaline Phosphatase buffer (200mM Tris-HCl pH8, 100 mM MgCl2) in a microtiter plate format (Thermofast, Advance Biotech, Polylabo n° 35 355). The reaction mixture was incubated 30 min. at 37°C, and denaturated 10 min. at 94°C afterwards.

4 Microsequencing

4.1 Microsequencing reaction conditions using 1 oligonucleotide primer, no PCR Passivation step, and using 2 color, 2 ddNTP detection mode

To each well containing 10µl of purified PCR product was added 10 µl of microsequencing reaction mixture containing 10 pmol microsequencing oligonucleotide (19mers, GENSET, crude synthesis, 5 OD), 1 U ThermoSequenase (Amersham, E79000G), 1.25 µl ThermoSequenase buffer (260 mM TrisHCl pH9.5, 65 mM MgCl2), and the two appropriate fluorescent ddNTPs corresponding to the polymorphic site, the final concentrations of the dyes are 15 nM (TAMRA-ddNTP NEN, NEL472 to NEL475; FAM-ddNTP NEN, NEL480 to NEL483). After 4 min. at 94°C, 20 PCR cycles of 15 sec. at 55°C, 5 sec. at 72°C and 10 sec. at 94°C were carried out in a Tetrad PTC-225 thermocycler (MJ Research). The microtiter plate was centrifuged 10 sec. at 1500 rpm (Heraeus, Megafuge 3.0 R).

This protocol has the advantage of reducing consumption of labeled ddNTP reagents but requires 6 different microsequencing mixtures, one for each polymorphisms.

4.2 Microsequencing reaction conditions using 1 oligonucleotide primer, no PCR Passivation step, and 2 color, 4 ddNTP detection mode

To each well containing 10µl of purified PCR product was added 10 µl of microsequencing reaction mixture containing 10 pmol microsequencing oligonucleotide (19mers, GENSET, crude synthesis, 5 OD), 1 U ThermoSequenase (Amersham, E79000G), 1.25 µl ThermoSequenase buffer (260 mM TrisHCl pH9.5, 65 mM MgCl2), and the four fluorescent ddNTPs (ddATP and ddUTP in

one color and ddCTP and ddGTP in another color), the final concentrations of the dyes are 15 nM (TAMRA-ddNTP NEN, NEL472 to NEL475; FAM-ddNTP NEN, NEL480 to NEL483). After 4 min. at 94°C, 20 PCR cycles of 15 sec. at 55°C, 5 sec. at 72°C and 10 sec. at 94°C were carried out in a Tetrad PTC-225 thermocycler (MJ Research). The microtiter plate was centrifuged 10 sec. at 1500 rpm (Heraeus, Megafuge 3.0 R).

This protocol has the advantage of allowing a single microsequencing mixture for four out of six polymorphisms representing 85% of all polymorphisms, but has the disadvantage of increasing the consumption of the labeled ddNTP reagents.

4.3 Microsequencing reaction conditions using 1 oligonucleotide primer, no PCR Passivation step, and 4 color, 4 ddNTP detection mode

To each well containing 10µl of purified PCR product was added 10 µl of microsequencing reaction mixture containing 10 pmol microsequencing oligonucleotide (19mers, GENSET, crude synthesis, 5 OD), 1 U ThermoSequenase (Amersham, E79000G), 1.25 µl ThermoSequenase buffer (260 mM TrisHCl pH9.5, 65 mM $MgCl_2$), and the four fluorescent ddNTPs , JOE-ddATP (NEL486), TAMRA-ddUTP (NEL472), ROX-ddCTP (NEL477) and FAM-ddGTP (NEL483) at a final concentrations of 15 nM. After 4 min. at 94°C, 20 PCR cycles of 15 sec. at 55°C, 5 sec. at 72°C and 10 sec. at 94°C were carried out in a Tetrad PTC-225 thermocycler (MJ Research). The microtiter plate was centrifuged 10 sec. at 1500 rpm (Heraeus, Megafuge 3.0 R).

This protocol has the advantage of having 1 microsequencing mixture for the six polymorphisms but the disadvantage of increasing the consumption of labeled ddNTP reagents and a more complex detection apparatus.

4.4 Microsequencing reaction conditions using 2 oligonucleotide primer, no PCR Passivation step, and 2 color, 4 ddNTP detection mode

To each well containing 10µl of purified PCR product was added 10 µl of microsequencing reaction mixture containing 10 pmol of both microsequencing oligonucleotide (one for each strand) (19mers, GENSET, crude synthesis, 5 OD), 1 U ThermoSequenase (Amersham, E79000G), 1.25 µl ThermoSequenase buffer (260 mM TrisHCl pH9.5, 65 mM $MgCl_2$), and the four fluorescent ddNTPs (ddATP and ddUTP in one color and ddCTP and ddGTP in another color), the final concentrations of the dyes are 15 nM (TAMRA-ddNTP NEN, NEL472 to NEL475; FAM-ddNTP NEN, NEL480 to NEL483). After 4 min. at 94°C, 20 PCR cycles of 15 sec. at 55°C, 5 sec. at 72°C and 10 sec. at 94°C were carried out in a Tetrad PTC-225 thermocycler (MJ Research). The microtiter plate was centrifuged 10 sec. at 1500 rpm (Heraeus, Megafuge 3.0 R).

This protocol has the advantage of allowing a single microsequencing mix for four out of six polymorphisms representing 85% of all polymorphisms as well as a more robust genotyping reaction eliminating the need of SNP optimization. This protocol has the disadvantage of increasing the

consumption of the labeled ddNTP reagents.

4.5 Microsequencing reaction conditions using 2 oligonucleotide primers, no PCR Passivation step, and 4 color, 4 ddNTP detection mode

To each well containing 10µl of purified PCR product was added 10 µl of microsequencing reaction mixture containing 10 pmol of both microsequencing oligonucleotide (one for each strand) (19mers, GENSET, crude synthesis, 5 OD), 1 U ThermoSequenase (Amersham, E79000G), 1.25 µl ThermoSequenase buffer (260 mM TrisHCl pH9.5, 65 mM $MgCl_2$), and the four fluorescent ddNTPs , JOE-ddATP (NEL486), TAMRA-ddUTP (NEL472), ROX-ddCTP (NEL477) and FAM-ddGTP (NEL483) at a final concentrations of 15 nM. After 4 min. at 94°C, 20 PCR cycles of 15 sec. at 55°C, 5 sec. at 72°C and 10 sec. at 94°C were carried out in a Tetrad PTC-225 thermocycler (MJ Research). The microtiter plate was centrifuged 10 sec. at 1500 rpm (Heraeus, Megafuge 3.0 R).

This protocol has the advantage of allowing a single microsequencing mixture for the six polymorphisms as well as a more robust genotyping reaction eliminating the need of SNP optimization, but has the disadvantage of increasing the consumption of the labeled ddNTP reagents and requiring a more complex detection apparatus.

4.6 Microsequencing reaction conditions using 1 oligonucleotide primer, PCR Passivation, and 2 color, 2 ddNTP detection mode

To each well containing 10µl of purified PCR product was added 10 µl of PCR passivation reaction mixture containing 1 U ThermoSequenase (Amersham, E79000G), 1.25 µl ThermoSequenase buffer (260 mM TrisHCl pH9.5, 65 mM $MgCl_2$), and 0.1 nM the four unlabeled ddNTPs. After 4 min. at 94°C, 10 PCR cycles of 15 sec. at 55°C, 5 sec. at 72°C and 10 sec. at 94°C were carried out in a Tetrad PTC-225 thermocycler (MJ Research). The microtiter plate was centrifuged 10 sec. at 1500 rpm (Heraeus, Megafuge 3.0 R).

To each well containing 20µl of passivated PCR product was added 10 µl of microsequencing reaction mixture containing 1.25 µl ThermoSequenase buffer (260 mM TrisHCl pH9.5, 65 mM $MgCl_2$),

10 pmol microsequencing oligonucleotide (19mers, GENSET, crude synthesis, 5 OD), and the two appropriate fluorescent ddNTPs corresponding to the polymorphic site, the final concentrations of the dyes are 15 nM (TAMRA-ddNTP NEN, NEL472 to NEL475; FAM-ddNTP NEN, NEL480 to NEL483). After 4 min. at 94°C, 20 PCR cycles of 15 sec. at 55°C, 5 sec. at 72°C and 10 sec. at 94°C were carried out in a Tetrad PTC-225 thermocycler (MJ Research). The microtiter plate was centrifuged 10 sec. at 1500 rpm (Heraeus, Megafuge 3.0 R).

This protocol has the advantage of reducing consumption of the expensive labeled ddNTP reagents, as well as reducing fluorescence background due to nonspecific incorporation of labeled ddNTPs in the PCR product, but requires 6 different microsequencing mixtures (one for each

polymorphism).

4.7 Microsequencing reaction conditions using 1 oligonucleotide primer, PCR Passivation, and 2 color, 4 ddNTP detection mode

To each well containing 10µl of purified PCR product was added 10 µl of PCR passivation reaction mixture containing 1 U ThermoSequenase (Amersham, E79000G), 1.25 µl ThermoSequenase buffer (260 mM TrisHCl pH9.5, 65 mM MgCl2), and 0.1 nM the four unlabeled ddNTPs. After 4 min. at 94°C, 10 PCR cycles of 15 sec. at 55°C, 5 sec. at 72°C and 10 sec. at 94°C were carried out in a Tetrad PTC-225 thermocycler (MJ Research). The microtiter plate was centrifuged 10 sec. at 1500 rpm (Heraeus, Megafuge 3.0 R).

To each well containing 20µl of passivated PCR product was added 10 µl of microsequencing reaction mixture containing 1.25 µl ThermoSequenase buffer (260 mM TrisHCl pH9.5, 65 mM MgCl2),

10 pmol microsequencing oligonucleotide (19mers, GENSET, crude synthesis, 5 OD), and the four fluorescent ddNTPs (ddATP and ddUTP in one color and ddCTP and ddGTP in another color), the final concentrations of the dyes are 15 nM (TAMRA-ddNTP NEN, NEL472 to NEL475; FAM-ddNTP NEN, NEL480 to NEL483). After 4 min. at 94°C, 20 PCR cycles of 15 sec. at 55°C, 5 sec. at 72°C and 10 sec. at 94°C were carried out in a Tetrad PTC-225 thermocycler (MJ Research). The microtiter plate was centrifuged 10 sec. at 1500 rpm (Heraeus, Megafuge 3.0 R).

This protocol has the advantage of allowing a single microsequencing mixture for four out of six polymorphisms, representing 85% of all polymorphisms, as well as reducing fluorescence background due to unspecific incorporation of labeled ddNTPs in the PCR product. The protocol has the disadvantage of increasing the consumption of the labeled ddNTP reagents.

4.8 Microsequencing reaction conditions (1 oligonucleotide primer, PCR passivation and 4 color, 4 ddNTP detection mode)

To each well containing 10µl of purified PCR product was added 10 µl of PCR passivation reaction mixture containing 1 U ThermoSequenase (Amersham, E79000G), 1.25 µl ThermoSequenase buffer (260 mM TrisHCl pH9.5, 65 mM MgCl2), and 0.1 nM the four unlabeled ddNTPs. After 4 min. at 94°C, 10 PCR cycles of 15 sec. at 55°C, 5 sec. at 72°C and 10 sec. at 94°C were carried out in a Tetrad PTC-225 thermocycler (MJ Research). The microtiter plate was centrifuged 10 sec. at 1500 rpm (Heraeus, Megafuge 3.0 R).

To each well containing 20µl of passivated PCR product was added 10 µl of microsequencing reaction mixture containing 1.25 µl ThermoSequenase buffer (260 mM TrisHCl pH9.5, 65 mM MgCl2),

10 pmol microsequencing oligonucleotide (19mers, GENSET, crude synthesis, 5 OD), and the four fluorescent ddNTPs , JOE-ddATP (NEL486), TAMRA-ddUTP (NEL472), ROX-ddCTP (NEL477)

and FAM-ddGTP (NEL483) at a final concentrations of 15 nM. After 4 min. at 94°C, 20 PCR cycles of 15 sec. at 55°C, 5 sec. at 72°C and 10 sec. at 94°C were carried out in a Tetrad PTC-225 thermocycler (MJ Research). The microtiter plate was centrifuged 10 sec. at 1500 rpm (Heraeus, Megafuge 3.0 R).

This protocol has the advantage of allowing a single microsequencing mixture for the six polymorphisms as well as reducing fluorescence background due to unspecific incorporation of labeled ddNTPs in the PCR product, but the disadvantage of increasing in the consumption of the labeled ddNTP reagents and a more complex detection apparatus.

4.9 Microsequencing reaction conditions (2 oligonucleotide primer, PCR passivation and 2 color, 4 ddNTP detection mode)

To each well containing 10µl of purified PCR product was added 10 µl of PCR passivation reaction mixture containing 1 U ThermoSequenase (Amersham, E79000G), 1.25 µl ThermoSequenase buffer (260 mM TrisHCl pH9.5, 65 mM $MgCl_2$), and 0.1 nM the four unlabeled ddNTPs. After 4 min. at 94°C, 10 PCR cycles of 15 sec. at 55°C, 5 sec. at 72°C and 10 sec. at 94°C were carried out in a Tetrad PTC-225 thermocycler (MJ Research). The microtiter plate was centrifuged 10 sec. at 1500 rpm (Heraeus, Megafuge 3.0 R).

To each well containing 20µl of passivated PCR product was added 10 µl of microsequencing reaction mixture containing 1.25 µl ThermoSequenase buffer (260 mM TrisHCl pH9.5, 65 mM $MgCl_2$),
10 pmol of both microsequencing oligonucleotide (one for each strand) (19mers, GENSET, crude synthesis, 5 OD), and the four fluorescent ddNTPs (ddATP and ddUTP in one color and ddCTP and ddGTP in another color), the final concentrations of the dyes are 15 nM (TAMRA-ddNTP NEN, NEL472 to NEL475; FAM-ddNTP NEN, NEL480 to NEL483). After 4 min. at 94°C, 20 PCR cycles of 15 sec. at 55°C, 5 sec. at 72°C and 10 sec. at 94°C were carried out in a Tetrad PTC-225 thermocycler (MJ Research). The microtiter plate was centrifuged 10 sec. at 1500 rpm (Heraeus, Megafuge 3.0 R).

This protocol has the advantage of allowing a single microsequencing mixture for four out of six polymorphisms representing 85% of all polymorphisms, a more robust genotyping reaction eliminating the need of SNP optimization as well as reducing fluorescence background due to nonspecific incorporation of labeled ddNTPs in the PCR product, but the disadvantage of increasing the consumption of the labeled ddNTP reagents.

4.10 Microsequencing reaction conditions (2 oligonucleotide primer, PCR passivation and 4 color, 4 ddNTP detection mode)

To each well containing 10µl of purified PCR product was added 10 µl of PCR passivation reaction mixture containing 1 U ThermoSequenase (Amersham, E79000G), 1.25 µl

ThermoSequenase buffer (260 mM TrisHCl pH9.5, 65 mM $MgCl_2$), and 0.1 nM the four unlabeled ddNTPs. After 4 min. at 94°C, 10 PCR cycles of 15 sec. at 55°C, 5 sec. at 72°C and 10 sec. at 94°C were carried out in a Tetrad PTC-225 thermocycler (MJ Research). The microtiter plate was centrifuged 10 sec. at 1500 rpm (Heraeus, Megafuge 3.0 R).

To each well containing 20µl of passivated PCR product was added 10 µl of microsequencing reaction mixture containing 1.25 µl ThermoSequenase buffer (260 mM TrisHCl pH9.5, 65 mM $MgCl_2$),

10 pmol of both microsequencing oligonucleotide (one for each strand) (19mers, GENSET, crude synthesis, 5 OD), and the four fluorescent ddNTPs , JOE-ddATP (NEL486), TAMRA-ddUTP (NEL472), ROX-ddCTP (NEL477) and FAM-ddGTP (NEL483) at a final concentrations of 15 nM. After 4 min. at 94°C, 20 PCR cycles of 15 sec. at 55°C, 5 sec. at 72°C and 10 sec. at 94°C were carried out in a Tetrad PTC-225 thermocycler (MJ Research). The microtiter plate was centrifuged 10 sec. at 1500 rpm (Heraeus, Megafuge 3.0 R).

This protocol has the advantage of allowing a single microsequencing mixture for the six polymorphisms, a more robust genotyping reaction eliminating the need of SNP optimization step as well as reducing fluorescence background due to nonspecific incorporation of labeled ddNTPs in the PCR product. This protocol has the disadvantage of increasing the consumption of the labeled ddNTP reagents and requiring a more complex detection apparatus.

5 Removal of the dyes

5.1 Removal of the unincorporated dye terminator

The unincorporated dye terminators were removed by gel filtration. 45µl/well of Sephadex G-50 fine dry powder (Pharmacia, ref 17-0573-02) was loaded on the MultiScreen Column Loader (Millipore, MACL 09645) and transferred into a MultiScreen microtiterplate (Millipore, MAGVN 2250). 300µl of TE 1X (10 mM Tris, 1mM EDTA) are added to every well containing the G50 Sephadex powder. The G50 Sephadex is then left to swell for 3 hours at room temperature. The G50 columns are rinsed twice with 50µl of TE 1X, by centrifugating after each buffer addition for 3 minutes at 2000 t/min (Heraeus, Megafuge 3.0 R). The 20 µl microsequencing reaction, are then carefully deposited on the top of the G50 columns. The Multiscreen microtiterplates are centrifuged 5 minutes at 2000t/min. The purified samples are recovered in the eluant fraction and transferred to a black, clear bottom, 384 microtiter plate (Greiner, REF 781096). The samples are directly analyzed after adjusting the volumes to 15µl.

5.2 Removal of the unincorporated dye terminator with internal control

Before removal of unincorporated dyes, 3µl of a 150 nM of a Texas Red solution was added to each well after the microsequencing reactions. The unincorporated dye terminators and the Texas

Red which serves as an internal control for the dye purification step were removed by gel filtration. 45µl/well of Sephadex G-50 fine dry powder (Pharmacia, ref 17-0573-02) was loaded on the MultiScreen Column Loader (Millipore, MACL 09645) and transferred into a MultiScreen microtiterplate (Millipore, MAGVN 2250). 300µl of TE 1X (10 mM Tris, 1mM EDTA) are added to every well containing the G50 Sephadex powder. The G50 Sephadex is then left to swell for 3 hours at room temperature. The G50 columns are rinsed twice with 50µl of TE 1X, by centrifugating after each buffer addition for 3 minutes at 2000 t/min (Heraeus, Megafuge 3.0 R). The entire microsequencing reaction, are then carefully deposited on the top of the G50 columns. The Multiscreen microtiterplates are centrifuged 5 minutes at 2000t/min. The purified samples are recovered in the eluant fraction and transferred to a black, clear bottom, 384 microtiter plate (Greiner, REF 781096). The samples are directly analyzed after adjusting the volumes to 15µl.

6 Fluorescence Detection

6.1 Two Color Direct Fluorescent Analysis on the Fluorimager (Molecular dynamics)

The fluorescent detection system used is the Fluorimager (Molecular Dynamics). An excitation laser of 488 nm and of 514 nm and an emission filter of 530 nm and 590 nm were used respectively for the Fam and Tamra fluorophores. The setup was the following: pixel size :100 micron, digital resolution : 16 bits, detection sensitivity: normal, PMT voltage: 750 Volts, acquisition mode : dual.

The raw data were processed in order to remove background and spectral contribution of the Fam in the Tamra channel. Two standard curves were preformed by using two fold serial dilutions of chemically modified and purified oligo-Fam and oligo-Tamra (GENSET, purified synthesis, 1 OD). Ten microliter of these dilution ranging from 2.5 fmole/µl to 0.04 fmole/µl were added to empty wells of the microtiter plate containing the samples. Using the standard curve, the incorporated Fam and Tamra fluorophores of every sample were quantified.

The Fam and Tamra quantities are then plotted per well and per polymorphic site, the sample distribution enables the determination of the genotype/sample.

6.2 Two Color Direct Fluorescent Analysis on the Analyst (LJL Biosystems)

The fluorescent detection system used is the Analyst (LJL Biosystems). A lamp is used as the excitation mean. Excitation filter of 490 nm and of 550 nm and an emission filter of 520 nm and 580 nm were used respectively for the Fam and Tamra fluorophores. The measurement mode used was an average of three reads per well using the SmartRead 2+ mode.

In some cases, the raw data were processed in order to remove. Two standard curves were preformed by using two fold serial dilutions of chemically modified and purified oligo-Fam and oligo-Tamra (GENSET, purified synthesis, 1 OD). Ten microliter of these dilution ranging from 2.5

fmole/µl to 0.04 fmole/µl were added to empty wells of the microtiter plate containing the samples. Using the standard curve, the incorporated Fam and Tamra fluorophores of every sample were quantified.

The Fam and Tamra quantities are then plotted per well and per polymorphic site, the sample distribution enables the determination of the genotype/sample.

Example 2

Homogenous Phase Direct Fluorescence Detection

The steps of sample preparation, PCR and PCR purification are carred out essentially as described above in Example 1. Subsequent fluorescence detection was also carried out essentially as in Example 1, except that excitation and emission filters for the dyes R110 and Tamra were used.

For microsequencing, reaction conditions with 1 oligonucleotide primer, no PCR Passivation step, and using 2 color, 2 ddNTP detection mode were used. To each well containing 10µl of purified PCR product was added 10 µl of microsequencing reaction mixture containing 10 pmol (0.5µM) microsequencing oligonucleotide (19mers, GENSET, crude synthesis, 5 OD), 1 U ThermoSequenase (Amersham, E79000G), 1.25 µl ThermoSequenase buffer (260 mM TrisHCl pH9.5, 65 mM MgCl2), and the two appropriate fluorescent ddNTPs corresponding to the polymorphic site, the final concentrations of the dyes are 18 nM (TAMRA-ddNTP and R110-ddNTP). 20 PCR cycles of 5 sec. at 55°C, 15 sec. at 72°C and 10 sec. at 94°C were carried out in a .Tetrad PTC-225 thermocycler (MJ Research). The microtiter plate was centrifuged 10 sec. at 1500 rpm (Heraeus, Megafuge 3.0 R). The samples were diluted (1:1 dilution) with a buffer (100mM KCL, 100mM Tris.HCl, 10mM Mg2CL and 16% Glycerol) and the fluorescence intensity was measured on the LJL instrument.

As described herein in Figure 2A and 2B, the total intensities of negative controls (T- without oligo and T- without DNA) were compared with the intensities of the sample in order to detect quenching due to the labeled ddNTP being incorporated into an oligonucleotide.

While the preferred embodiment of the invention has been illustrated and described, it will be appreciated that various changes can be made therein by the one skilled in the art without departing from the spirit and scope of the invention. All documents cited herein are incorporated herein by reference in their entirety.

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What is claimed:

1. A method for the detection of a target nucleotide in a nucleic acid sample comprising:

(a) Providing a nucleic acid sample comprising a target nucleic acid wherein the nucleotide bases spanning said target nucleic acid are unpaired;

(b) contacting said sample with an oligonucleotide primer capable of hybridizing specifically to a stretch of nucleotides bases present in said sample 3' to said target nucleic acid;

(c) subjecting a hybrid formed in step (b) to a single nucleotide template-dependent primer extension reaction in the presence of a chain terminating nucleotide comprising a fluorescent label, wherein said nucleotide is capable of being incorporated sequence specifically into the primer; and

(d) detecting the incorporation of a labeled nucleotide in the primer by measuring direct fluorescence intensity.

2. A method for the detection of a target nucleotide in a nucleic acid sample comprising:

(a) Providing a nucleic acid sample comprising a target nucleic acid, said nucleic acid sample having a first strand and a second strand complementary thereto, wherein the nucleotide bases spanning said target nucleic acid are unpaired;

(b) contacting said sample with a first oligonucleotide primer capable of hybridizing specifically to a stretch of nucleotides bases present in said first strand of sample 3' to said target nucleic acid;

(c) contacting said sample with a second oligonucleotide primer capable of hybridizing specifically to a stretch of nucleotides bases present in said second strand of sample 3' to said target nucleic acid;

(d) subjecting a hybrid formed in steps (b) and (c) to a single nucleotide template-dependent primer extension reaction in the presence of at least two different chain terminating nucleotides, wherein each type of nucleotide comprises a distinct fluorescent label and is capable of being incorporated sequence specifically into the primer;

(e) detecting the incorporation of a labeled nucleotide in the primer.

3. A method for the detection of a target nucleotide in a nucleic acid sample comprising:

(a) Providing a nucleic acid sample comprising a target nucleic acid;

(b) treating said nucleic acid sample with unlabeled nucleotides in the presence of a

polymerase; and

(c) conducting a template directed primer extension assay, thereby detecting a target nucleotide in said sample.

4. A method for the detection of a target nucleotide in a nucleic acid sample according to claim 3, wherein said step of conducting a template directed primer extension assay comprises:

(a) Providing a nucleic acid sample comprising a target nucleic acid;

(b) contacting said sample with an oligonucleotide primer capable of hybridizing specifically to a stretch of nucleotides bases present in said sample;

(c) subjecting a hybrid formed in step (b) to a nucleic acid template-dependent primer extension reaction in the presence of a nucleotide comprising a fluorescent label, wherein said nucleotide is capable of being incorporated sequence specifically into the primer; and

(d) detecting the incorporation of a labeled nucleotide in the primer.

5. A method for the detection of a target nucleotide in a nucleic acid sample according to claim 4, wherein an oligonucleotide primer is capable of hybridizing 3' to a target nucleotide in said nucleic acid sample.

6. A method for the detection of a target nucleotide in a nucleic acid sample according to claim 1, 2 or 5, wherein an oligonucleotide primer is capable of hybridizing immediately 3' to a target nucleotide in said nucleic acid sample.

7. A method for the detection of a target nucleotide in a nucleic acid sample according to claim 1, wherein said step of measuring direct fluorescence intensity comprises measuring fluorescence quenching.

8. A method for the detection of a target nucleotide in a nucleic acid sample according to claim 1 or 7, wherein a decrease in direct fluorescence intensity is measured, said decrease indicating the incorporation of a labeled nucleotide into an oligonucleotide primer.

9. A method for the detection of a target nucleotide in a nucleic acid sample according to claim 2 to 4, wherein the incorporation of said nucleotide is detected by measuring direct fluorescence intensity.

10. A method for the detection of a target nucleotide in a nucleic acid sample according

to claim 2 to 4 wherein the incorporation of said chain terminating nucleotide is detected by measuring fluorescence polarization.

11. A method for the detection of a target nucleotide in a nucleic acid sample according to any one of claims 1 to 10, wherein said nucleic acid template-dependent primer extension reaction is carried out in the absence of dATP, dTTP, dGTP or dCTP.

12. A method for the detection of a target nucleotide in a nucleic acid sample according to any one of claims 1 to 11 wherein said chain terminating nucleotides comprise fluorescently labeled ddATP, ddTTP, ddGTP and ddCTP.

13. A method for the detection of a target nucleotide in a nucleic acid sample according to claim 12, wherein each type of chain terminating nucleotide comprises a distinct fluorescent label.

14. A method for the detection of a target nucleotide in a nucleic acid sample according to claim 13, wherein ddATP and ddTTP are labeled with a first fluorescent dye and ddCTP and ddGTP are labeled with a second fluorescent dye.

15. A method for the detection of a target nucleotide in a nucleic acid sample according to any one of claims 1 to 11, wherein said chain terminating nucleotides comprise a first nucleotide selected from the group consisting of ddATP and ddTTP labeled with a first fluorescent dye, and a second nucleotide selected from the group consisting of ddCTP and ddGTP labeled with a second fluorescent dye.

16. A method for the detection of a target nucleotide in a nucleic acid sample according to claims 12 to 15, wherein said fluorescently labeled nucleotides are provided as a mixture.

17. A method for the detection of a target nucleotide in a nucleic acid sample according to any one of the above claims, wherein fluorescent labels comprise TAMRA and FAM dyes.

18. A method for the detection of a target nucleotide in a nucleic acid sample according to any one of the above claims, comprising providing a control molecule labeled with a fluorescent dye distinct from dyes used to label nucleotides capable of being incorporated into said oligonucleotide primer, whereby said control molecule as an internal control for fluorescence intensity.

19. A method for the detection of a target nucleotide in a nucleic acid sample according to any one of claims 1 to 6 and 8 to 18, wherein said nucleic acid sample comprises at least one single nucleotide polymorphism.

20. A method for the detection of a target nucleotide in a nucleic acid sample according to any one of claims 1 to 6 and 8 to 19, further comprising separating unincorporated labeled nucleotides from oligonucleotide primers extended by said nucleic acid template-dependent primer extension reaction prior to detection of fluorescence.

21. A method for the detection of a target nucleotide in a nucleic acid sample according to claim 20, wherein said separating step comprises gel filtration.

22. A method for the detection of a target nucleotide in a nucleic acid sample according to any one of the above claims, wherein oligonucleotide primers are spatially separated.

23. A method for the detection of a target nucleotide in a nucleic acid sample according to any one of the above claims, wherein an oligonucleotide primer or a nucleic acid sample is attached to a solid support.

24. A method for the detection of a target nucleotide in a nucleic acid sample according to claim 23, wherein said solid support comprises an addressable oligonucleotide array.

25. A method for the detection of a target nucleotide in a nucleic acid sample comprising:

(a) performing a nucleic acid amplification reaction on a sample nucleic acid,

(b) purifying said nucleic acid sample, and

(c) detecting a target nucleotide according to any one of the above claims.

26. A method for the detection of a target nucleotide in a nucleic acid sample according to claim 25, wherein said purification comprises removing amplification primers and dNTPs.

27. A method for the detection of a target nucleotide in a nucleic acid sample according to claim 25 , wherein said purification step comprises treating a nucleic acid sample with at least one enzyme.

28. A method for the detection of a target nucleotide in a nucleic acid sample according to claim 27, wherein said enzyme comprises shrimp alkaline phosphatase.

29. A method for determining the genotype of a selected organism at one or more genetic loci comprising:

(a) obtaining from the organism a sample containing genomic DNA

(b) identifying the target nucleotide bases present at each of the one or more polymorphic sites of interest according to a nucleic acid detection method of any one of the above claims, and

(c) determining the genotype of said organism at one or more genetic loci based on the different alleles identified in step (b).

30. A method of detecting an association between a genotype and a trait, comprising:

(a) determining the frequency of at least one biallelic marker in trait positive population;

(b) determining the frequency of at least one biallelic marker in a control; and

(c) determining whether a statistically significant association exists between said genotype and said trait, wherein at least one of said steps for determining the frequency of a biallelic marker comprises detecting a target nucleotide according to a nucleic acid detection method of any one of the above claims.

31. A method for the detection of a target nucleotide in a nucleic acid sample according to any one of claims 1 to 6 and 8 to 17, comprising providing, after said primer extension reaction is carried out, a fluorescent molecule comprising a fluorescent dye distinct from those used in the nucleic acid template dependent primer extension reaction, whereby said control fluorescent molecule serves as an internal control for removal of unincorporated labeled ddNTPs from extended oligonucleotide primers.

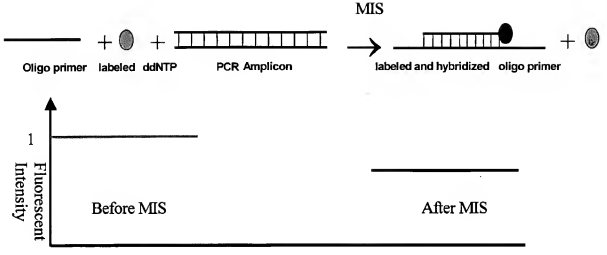
MIS
Oligo primer
labeled ddNTP
PCR Amplicon
labeled and hybridized oligo primer
1
Fluorescent Intensity
Before MIS
After MIS

Figure 1

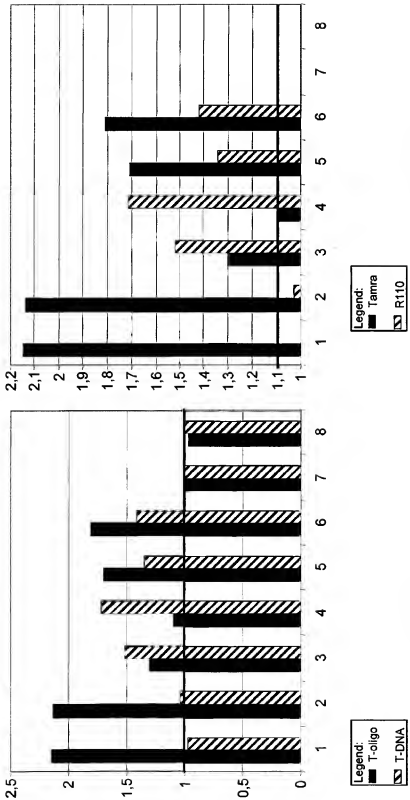

FIGURE 2B

FIGURE 2A

OLIGO 1

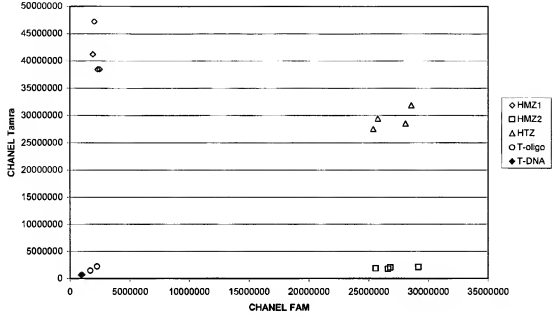

FIGURE 3A

OLIGO 2

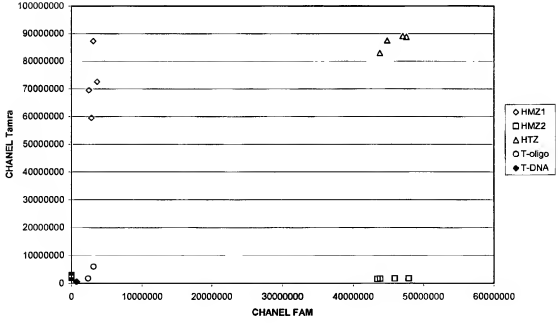

FIGURE 3B